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Quantitation of the Monoclonal Antibody Rituximab Using Volumetric Absorptive Microsampling, Impact-Assisted Extraction, Trypsin Digestion and LC-MRM

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OVERVIEW

PURPOSE

To demonstrate the applicability of volumetric absorptive microsampling (VAMS) for the quantitation of biotherapeutic monoclonal antibodies using a bottom-up LC-MRM approach.

METHOD

Human whole blood fortified with Rituximab was sampled onto Mitra® microsamplers (Neoteryx), dried at room temperature in the presence of desiccant, and extracted using impact-assisted extraction (IAE). Proteins were reduced, alkylated, and digested with trypsin. Data was acquired using a SCIEX Triple Quad 6500⁺ operated in (+)ESI-MRM.

RESULTS

Optimal Rituximab recovery was obtained using an ammonium bicarbonate buffer containing 20% acetonitrile and 0.25% octyl ß-glucopyranoside. The rituximab quantitation was unaffected by hematocrit (HCT) levels ranging from 0% (plasma) to 63%. Intra-day precision of the assay was <5.3% with accuracies between 96.8 and 105.7% for all QCs.

INTRODUCTION

VAMS has emerged as an alternative approach for blood sampling during clinical and nonclinical studies. It enables precise and accurate collection of a determined blood volume, therefore reducing the hematocrit effect associated with the dried blood spot (DBS) technique. Nevertheless, the sample hematocrit level can still bias drug measurements by affecting the desorption of the analytes from the VAMS device. The recently established IAE approach has proven to overcome such bias for small molecules and peptides, but its applicability to biotherapeutic proteins quantitation has yet to be verified. The usefulness of this approach for the bottom-up LC-MRM quantitation of the monoclonal antibody Rituximab in human blood is herein demonstrated.

Figure 1. Neoteryx Mitra® volumetric absorptive microsampling (VAMS)

METHODS

SAMPLE PREPARATION

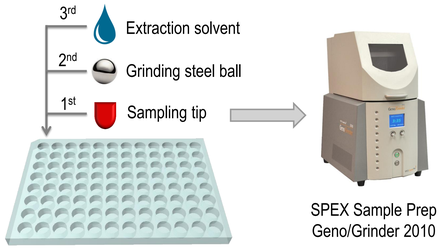

Figure 2. Impact Assisted Extraction (IAE)

Load Mitra® sampler into 96 well plate
Add grinding steel ball and extraction buffer
Seal plate and mix aggressively
Reduce (TCEP) and alkylate (IAM)
Digest with Trypsin (2 hours, 37°C)
Quench with formic acid
Analyze by LC-MRM on SCIEX Triple Quad 6500⁺

Figure 3. Rituximab Sample Processing

CHROMATOGRAPHY

- XBridge® Peptide BEH C_{18} (50 x 2.1 mm, 3.5 µm)
- Gradient elution with 0.2% acetic acid in water and ACN

DETECTION

- SCIEX Triple Quad 6500⁺ operated in (+) ESI-MRM mode

Table 1. Detection parameters for Rituximab surrogate peptide and stable isotopically labeled (SIL) peptide internal standard

Peptide Sequence	MRM Transition (*m/z*)	CE (V)
GLEWIGAIYPGNGDTSYNQK	728.6 > 590.8 (+3, y_{11}+2)	25
GLEWIGAIYPGNGDTSYNQK($^{13}C_6$, $^{15}N_2$)	731.3 > 594.8 (+3, y_{11}+2)	25

RESULTS

RITUXIMAB SAMPLE PROCESSING

Optimization of the extraction conditions included screening of multiple solvent mixtures to achieve optimal desorption of Rituximab from the Mitra® substrate. Protein solubility and compatibility with tryptic digestion were the primary drivers in the selection of extraction solvent. Optimal Rituximab recovery was obtained using a mixture of ammonium bicarbonate containing 20% acetonitrile and 0.25% octyl beta-glucopyranoside (Figure 4).

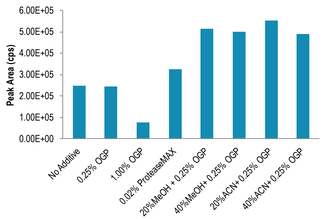

Figure 4. Extraction of Rituximab from VAMS using various extraction buffers. All buffers tested included 50mM ammonium bicarbonate supplemented with additives. OGP: octyl β-glucopyranoside.

The rate of desorption for Rituximab from the Mitra® substrate was found to be much slower than that observed for typical small molecules applications. However, extraction of Rituximab was greatly improved when the Mitra® substrate remained in solution during the entire sample processing procedure, including reduction with TCEP, alkylation with iodoacetamide and trypsin digestion (Figure 5).

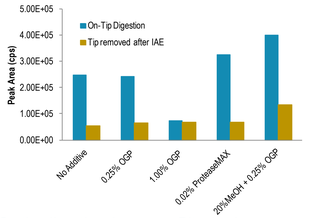

Figure 5. On-tip digestion improves Rituximab recovery from Mitra® substrate. All buffers tested included 50mM ammonium bicarbonate supplemented with additives. OGP: octyl β-glucopyranoside.

HEMATOCRIT EFFECT

Rituximab recovery using IAE was independent of blood HCT as indicated in Table 6 with yields ranging from 95.6 to 104% across all levels (Figure 6).

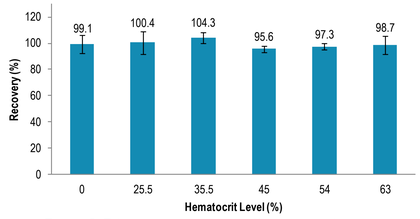

Figure 6. Rituxmab recovery at different hematocrit levels

LINEARITY, PRECISION AND ACCURACY

A precision and accuracy analytical batch was assayed using the optimal conditions described above. The assay was linear (weighted $1/x^2$) from 1.00 to 400.00 µg/mL with an LLOQ S:N > 10 (Figure 7 and Figure 8). Intra-day precision of the assay was < 5.3% with accuracies between 96.8 and 103.6% for all QCs.

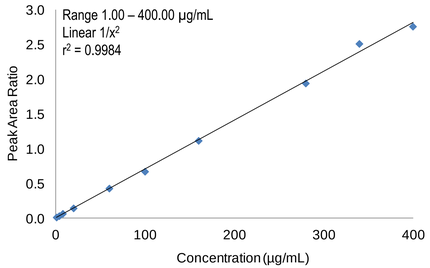

Figure 7. Calibration curve of Rituximab extracted from Mitra®

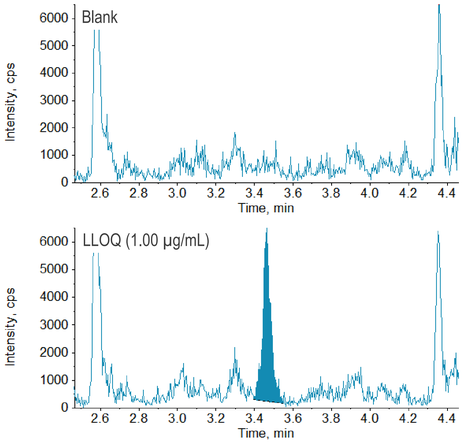

Figure 8. Representative chromatograms of Rituximab blank and LLOQ extracted from Mitra® (surrogate peptide monitored)

Table 2. Precision and accuracy of Rituximab extracted from Mitra®

	LOQ QC 1.00 µg/mL	Low QC 4.00 µg/mL	Mid QC 125.00 µg/mL	High QC 300.00 µg/mL
% Nominal	102.6	96.8	103.6	99.8
% CV	2.7	3.3	5.3	4.8

CONCLUSION

The applicability of VAMS combined with IAE was illustrated for the quantitation of the monoclonal antibody Rituximab using a bottom-up LC-MRM approach. The developed assay demonstrated excellent linearity, precision and accuracy whilst negating hematocrit effect.