Monitoring of Sequence Variants by MAM using High Resolution Mass Spectrometry

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INTRODUCTION

Sequence Variant Analysis (SVAs) is a term covering unintentional amino acid substitutions which can occur during protein biosynthesis. The term also covers amino acid deletions or insertions. As production of biotherapeutics is through living organisms, there are a variety of different places where the production of the protein from DNA to mRNA to protein can have a low level of infidelity. This level is approximately 10^{-9} per base pair for DNA replication and 10^{-4} – 10^{-5} per codon for protein translation. These variants contribute to the already complex heterogeneity of recombinant proteins and biotherapeutics and need to be characterized along with the biotherapeutics as these variants may cause immunogenic responses or decrease the safety and efficacy of the biotherapeutic.

The use of LC-MS/MS for profiling SVAs using peptide mapping is becoming more common as sensitivity and linear dynamic range of the instruments allows for the detection of low level variants, along with the for identification of the location of the amino acid substitution(s) in a matrix consisting of a high concentration of the biotherapeutic. Here we report the use of the TripleTOF ® 6600 system for identification of low abundant SVAs through Data-Dependent Acquisition (DDA) which is then imported into BioPharmaView™ software for monitoring of the variants using routine methodologies.

MATERIALS AND METHODS

Peptide Mapping Sample Preparation:
100 µg of NIST monoclonal antibody (mAb) reference standard (NIST, Gaithersburg, MD, USA) was diluted to 1 µg/µl with denaturing buffer (8M Urea in 100 mM Tris HCl pH 7.8). Sample is reduced with 100 mM DTT (Sigma-Aldrich, Dorset, UK) and alkylated with 100 mM Iodoacetamide (Sigma-Aldrich, Dorset, UK) before buffer exchanged with 10 mM Tris HCl pH 7.8 using BioSpin-6 column (Bio-Rad Laboratories, Watford, UK).

HPLC Conditions:
An ExionLC™ AD system with a Phenomenex Luna® Omega 1.6 µm PS C18 100Å, 150 x 2.1 mm column (Macclesfield, UK) at 45° C with a 120 min gradient of eluent A (water + 0.1% formic acid) and eluent B (acetonitrile + 0.1 % formic acid). A total of 10 µg digested trastuzumab was loaded on to the column.

A second set of injections was run using a 70 min gradient with 2 ug loading onto the column.

MS/MS Conditions:
A TripleTOF® 6600 LC-MS/MS system with an IonDrive™ source and in positive ion mode was used. Data was acquired using Data-Dependent Acquisition (DDA) to generate a library of SVAs to input into BioPharmaView™ software. DDA acquisition used a TOF-MS acquisition scan of 150 ms and 15 MSMS acquisitions at 40 ms per cycle. For Data Independent Analysis (DIA) using SWATH® acquisition a TOF-MS acquisition scan of 150 ms followed by SWATH® acquisition using 25 variable windows at 30 ms each was used.

Data Processing

Library Generation using ProteinPilot™
DDA data was combined and searched using ProteinPilot™ software using an emphasis on IgG and SVA modifications. The library used to search against consisted of the NIST sequence and the CHO fasta library combined. Data was manually checked and a list of SVAs was identified.

BioPharmaView™ Processing
NIST sequence and modifications were input into BioPharmaView™ software. The list of SVAs was input in as a set of impurities and calculations were created based on the SVA and the original tryptic peptide sequence. A percent calculation was created to determine the level of the SVA compared to the original sequence.

RESULTS

The NIST mAb digest was injected in triplicates at a concentration of 10 µg to increase the intensity of the SVAs which are considered to be low abundance in the samples. The triplicate injections were searched together in ProteinPilot™ software to ensure that the maximum number of SVAs were identified. This data was then manually filtered and the corresponding list was compared to the NIST SVA chapter to confirm at least 20 tryptic peptide variants.[1]

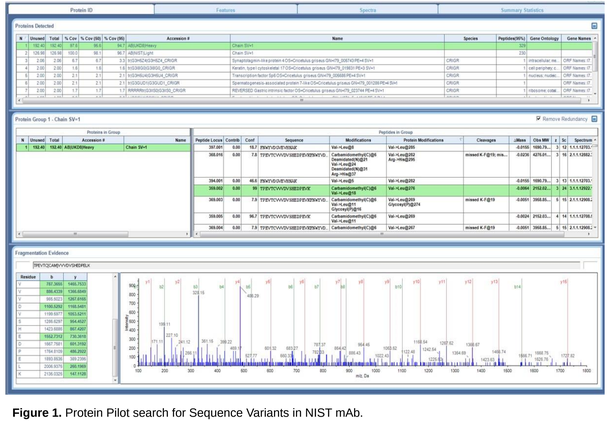

Figure 1. Protein Pilot search for Sequence Variants in NIST mAb.

20 of the sequence variants which corresponded to the same listed in the NIST chapter were input into BioPharmaView™ software and set as a fifth chain in the Sequence Features tab Figure 2). All SVAs used in this are tryptic based peptides. Calculation for SVAs can be seen in Figure 3 which calculated the SVA as a % of the total peptide.

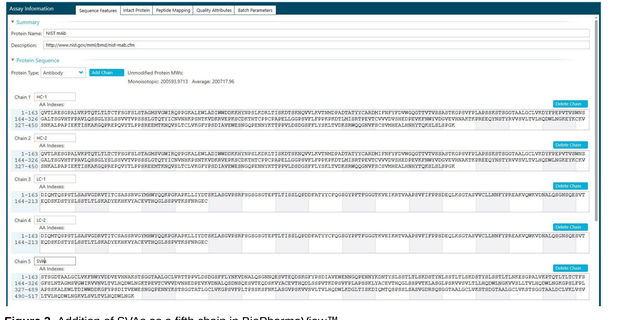

Figure 2. Addition of SVAs as a fifth chain in BioPharmaView™.

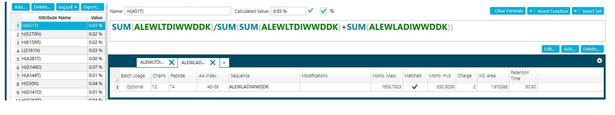

Figure 3. Setup of SVAs as % calculations in BioPharmaView™.

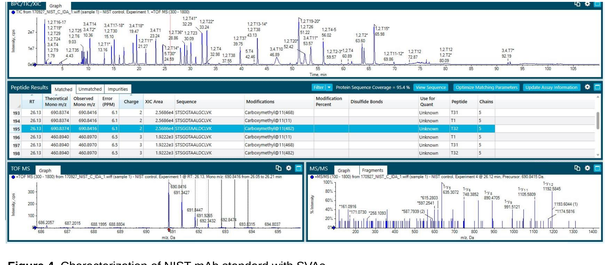

Figure 4. Characterization of NIST mAb standard with SVAs.

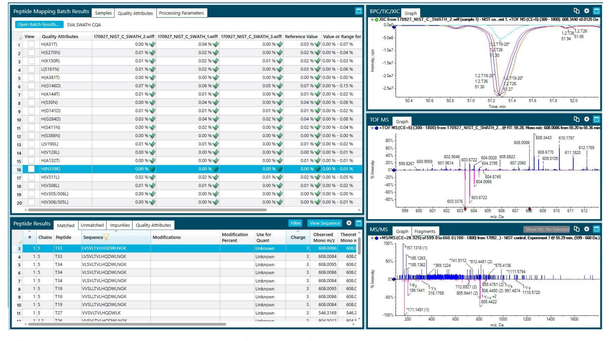

Figure 5. Results from triplicate analysis of SVAs using SWATH® acquisition.

Using % calculation of SVA, a list of results was created (Figure 5). Some of the calculations showed 0% as the final result, however, further scrutiny and XIC of the raw data (Figure 5, right side) showed a very small signal for the SVA (top right mirror plot). Signal for the peptide is of such low abundance that the signal can only be seen when using relative % in the mirror plot, and the level of the peptide is lower than what was originally set in the attribute tab.

CONCLUSIONS

Using the full strength of the TripleTOF ® 6600 system for it's sensitivity as well as it's linear dynamic range, data was acquired and searched with ProteinPilot™ software. From the data, 20 tryptic peptide sequence variants from the NIST SVA chapter were identified from 10 µg DDA over a 3 hour gradient. This was then input into BioPharmaView™ and the triplicate SWATH® acquisition data was processed to identify the SVAs in a short gradient with less sample injected. All 20 of the NIST SVAs from the chapter were identified, even at low abundance, showing the identification and analysis of SVAs using the SCIEX workflows.

REFERENCES

1. **Sequence Variants and Sequence Variant Analysis in Biotherapeutic Proteins**
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