Application Note

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Optimizing Adeno-Associated Virus Loading Amounts for Host Cell Protein Analysis

Using the Agilent AdvanceBio Peptide Plus column and the Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

An increasing number of pharmaceutical companies are investing in cell and gene therapy strategies. These therapeutics require assays to measure critical quality attributes for drug efficacy and safety, and some of these assays are similar to traditional biotherapeutics. One such assay is host cell protein (HCP) analysis that identifies and quantifies low-level proteins coproduced in the cell culture alongside the main therapeutic. Liquid chromatography/mass spectrometry (LC/MS) is a popular method for HCP analysis. To detect low-level host cell proteins by LC/MS, it is necessary to inject large amounts of therapeutic, and sometimes over 100 µg is required. The production of adeno-associated virus (AAV) has a notoriously low yield, which complicates HCP analysis. In this application note, a balance between AAV loading amount and host cell protein detection levels is explored.

Introduction

Host cell proteins are process-related impurities that result from cell culture production. Even after purification, low levels of some contaminant proteins remain. Host cell proteins may have adverse effects on the stability of the final product or may even cause immune responses. The accepted rule for the upper limit of HCPs is 100 ppm. However, guidance from regulatory agencies such as the FDA and European Medicines Agency do not provide specific values. The FDA's industry guidance for human gene therapy directed at neurodegenerative diseases recommends that residual host cell protein levels "be as low as can be reasonably achieved based on manufacturing experience."[1]

ELISA is the long-standing gold standard for HCP quantitation, but the assay cannot identify individual proteins. Detection with mass spectrometry is an increasingly prevalent orthogonal technique that allows for identification and relative quantitation of HCPs. A successful HCP workflow with LC/MS consists of an LC column that can handle large mass loads and retain sharp peaks with MS-compatible mobile phase modifiers and a detector with a wide, in-spectrum dynamic range. This workflow features the Agilent AdvanceBio Peptide Plus column for high mass loads and optimal peak shape with formic acid, coupled to the Agilent AdvanceBio 6545XT LC/Q-TOF system.

Experimental

Instrumentation

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

Reagents and chemicals

AAV1 empty reference standards were purchased from Charles River Labs. Digestion reagents and standard proteins were purchased from MilliporeSigma. Lys-C/trypsin was purchased from Promega Corporation. All mobile phase solvents and mobile phase additives were LC/MS grade.

Sample preparation

The AAV1 empty reference standards had a titer of 4.75×10^{12} VP/mL and a total volume of 100 µL. The AAV sample was denatured with trifluoroethanol, reduced and alkylated with dithiothreitol and iodoacetamide, digested overnight at 37 °C with mass spectrometry grade Lys-C/trypsin mix, and quenched with formic acid. To keep injection volumes reasonable, the digested sample was dried down and reconstituted in the starting mobile phase. To relatively quantify the host cell proteins, protein digests from nonhuman organisms were spiked into each sample and digested in the same manner as the AAV sample. Lysozyme was spiked at approximately 100 ppm and cytochrome C was spiked at approximately 10 ppm. Three LC/MS injections with different volumes/amounts were run. AAV titer and concentration do not have an exact conversion, therefore the most accurate way to discuss amounts injected are 50%, 33%, and 12.5% of the total amount of protein. To simplify naming in the results section, Table 1 summarizes the injection amounts.

Table 1. Injections and descriptions.

Injection Name	Injection Description
Injection A	Injection A was 50% of 4.75×10^{12} VP/mL, for a total of 2.38×10^{11} VP.
Injection B	Injection B was 33% of 4.75×10^{12} VP/mL, for a total of 1.58×10^{11} VP.
Injection C	Injection C was 12.5% of 4.75×10^{12} VP/mL, for a total of 5.94×10^{10} VP.

LC/MS analysis

LC/MS analysis was performed on a 1290 Infinity II LC coupled to a 6545XT AdvanceBio LC/Q-TOF with a dual Agilent Jet Stream source. Agilent MassHunter acquisition software for LC/MS systems 10.1 software was tuned and calibrated with SWARM autotune, and reference mass was used to calibrate throughout the runs. The Auto MS/MS algorithm was used to collect the data. Each dataset was processed with Byos software from Protein Metrics and the human proteome database was downloaded from SwissProt. LC parameters are displayed in Table 2 and LC/Q-TOF parameters are displayed in Table 3.

Table 2. LC/Q-TOF parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Plus 2.1 × 150 mm (p/n 695775-949)
Column Temperature	50 °C
Mobile Phase A	Water, 0.1% formic acid
Mobile Phase B	Acetonitrile 0.1% formic acid
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0 to 3 3 3 to 90 3 to 40 90 to 93 40 to 90 93 to 95 90 95 to 96 90 to 3
Post Time	3 minutes

Table 3. MS parameters.

Parameter	Value
Source	Dual Agilent Jet Stream
Dry Gas Temperature and Flow	325 °C and 13 L/min
Nebulizer	35 psig
Sheath Gas Temperature and Flow	275 °C and 12 L/min
Capillary Voltage	4,000 V
Nozzle	0 V
Fragmentor	175 V
Acquisition Rate	5/3 spectra/sec for MS and MS/MS
Reference Masses	121.0509, 922.0098

Results and discussion

Dynamic range and sensitivity

TOF- and Q-TOF-MS instruments have a wide in-spectrum dynamic range of up to five orders of magnitude, which allows for the detection of low-abundant species, even in complex biological samples. The ability to detect, identify, and characterize low-abundant species regardless of acquisition rate is crucial in host cell protein analysis, where the species of interest are inherently lower than the major components. Figure 1 shows an example of high analytical sensitivity with a low-abundant, although well-characterized peptide. The mass error of the precursor ion is less than 1 ppm. Despite being a low-abundant peptide, 100% sequence coverage of the peptide was attained, and the fragments are well above the noise level. Figure 2 shows two peptides coeluting. The first peptide is abundant and comes from the AAV 1 capsid proteins. The second peptide comes from the lowest host cell protein detected, bromodomain adjacent to zinc finger domain protein 2B. Bromodomain is more than 30 times less abundant than the capsid peptide. Despite the large load (50% of the original titer), the capsid peptide has a sharp peak with a tailing factor of 1.08.

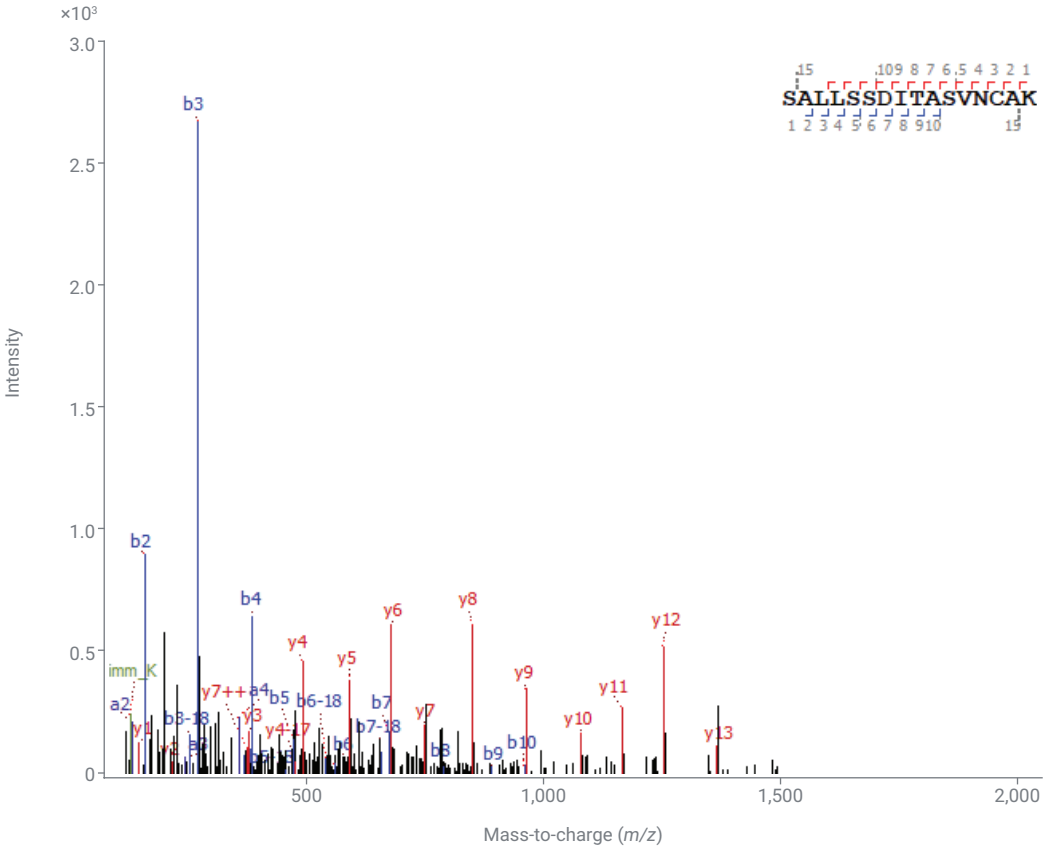

Figure 1. A low-abundant peptide is well-characterized due to the Q-TOF's high analytical sensitivity.

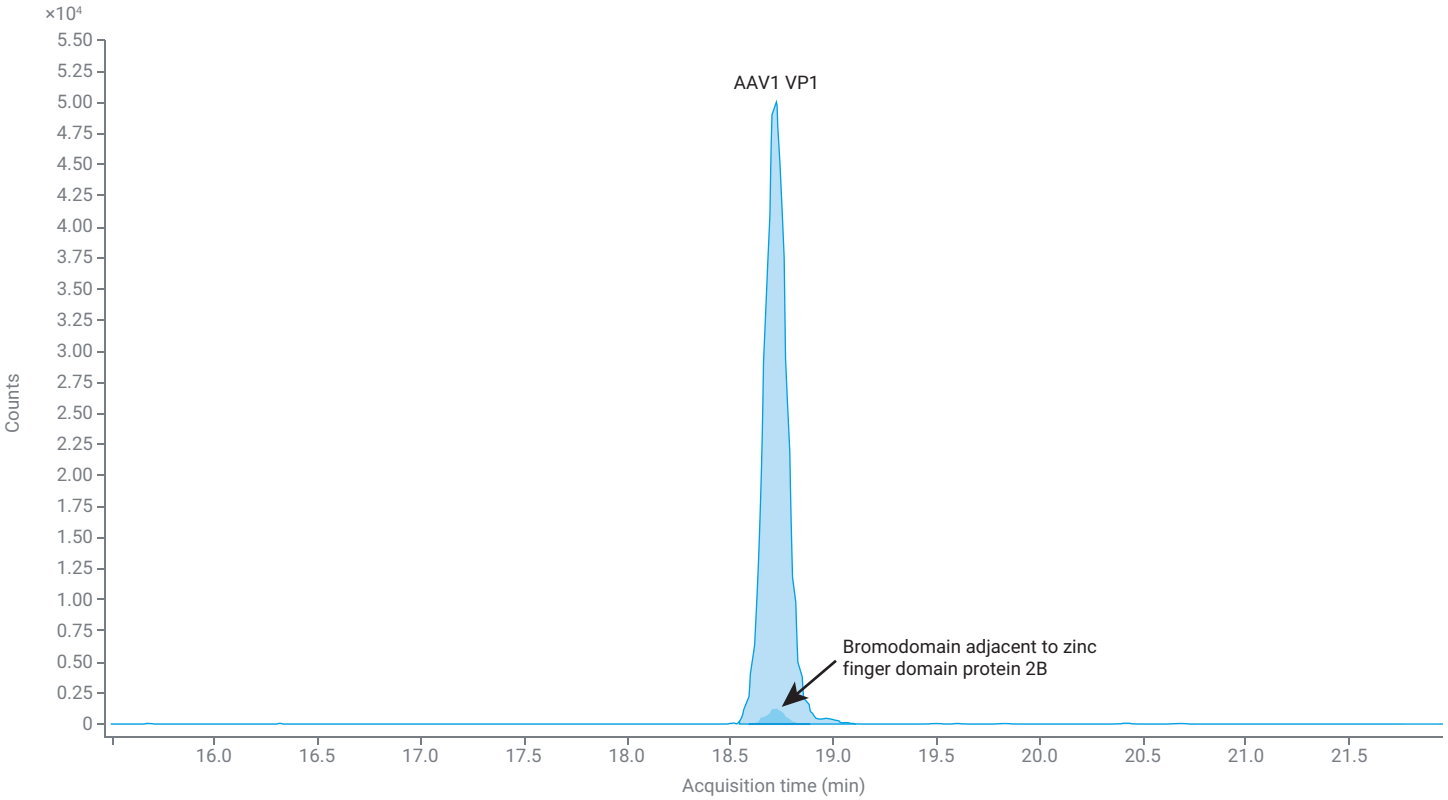

Figure 2. An AAV peptide co-eluting with a low-abundant peptide from an HCP, quantified under 1 ppm.

Loading comparison

Given the low yield of AAV capsids and the number of assays needed for critical quality attribute determination, samples must be aliquoted carefully. The goal of this application note was to assess sensitivity and the number of host cell proteins identified in comparison to the amount of sample injected. Less than 100 µg total was available for all three injections. Table 4 displays a list of host cell proteins and their relative amount in Injection A.

Table 4. List of HCPs and relative amounts in Injection A.

Host Cell Protein Name	Measured ppm	Detected in Injection B (Yes/No)
Zinc Finger Protein 512B	22.7	Yes
Protein FAM90A1	8.4	Yes
Ser/Thr Protein Kinase Nek4	5.9	Yes
(Lyso)-N-acylphosphatidylethanolamine Lipase	2.7	No
Leucine-rich PPR Motif-containing Protein	1.8	No
THAP Domain-containing Protein 5	0.9	No
KH Homology Domain-containing Protein 4	0.9	Yes
Unconventional Myosin-XV	0.7	No
Multidrug Resistance-associated Protein 1	0.7	Yes
Kinesin-like Protein KIF20B	0.4	No
Bromodomain Adjacent to Zinc Finger Domain Protein 2B	0.3	No

Because the samples were commercially available, purified reference standards, it is not surprising that the overall number of host cell proteins found was low. Although the overall number of proteins identified was small, the assay could detect proteins as low as 300 ppb in Injection A. Injection B could detect proteins as low as 500 ppb, while Injection C did not detect any HCPs that passed the score cutoff. Injections A and B are suitable for detecting less than the generally accepted concentration of 1 ppm. As summarized in Figure 3, Injection A detected six additional proteins not detected in Injection B. Injection B detected two host cell proteins, uncharacterized protein C7orf31 and deubiquitinating protein VCPIP1 that were not detected in Injection A.

The host cell proteins observed in the AAV samples did not have any homologous proteins on the high-risk HCPs list.[2] HCPs on the high-risk list are proteins that are immunogenic or are biologically or enzymatically active and have the potential to degrade the major product or excipients in the formulation buffer. While there are several lipases on the high-risk list and a single lipase was identified in Table 3, it is not on the high-risk list. The lipases on the high-risk list can have an impact on formulation by degrading polysorbates that are composed of fatty acid mono- and multi-esters. AAV tends to avoid polysorbate 20/80 as a surfactant. Instead, AAV uses poloxamer, a surfactant that is composed of poly (propylene oxide) and poly (ethylene oxide) chains and should not be affected by the lipase observed.

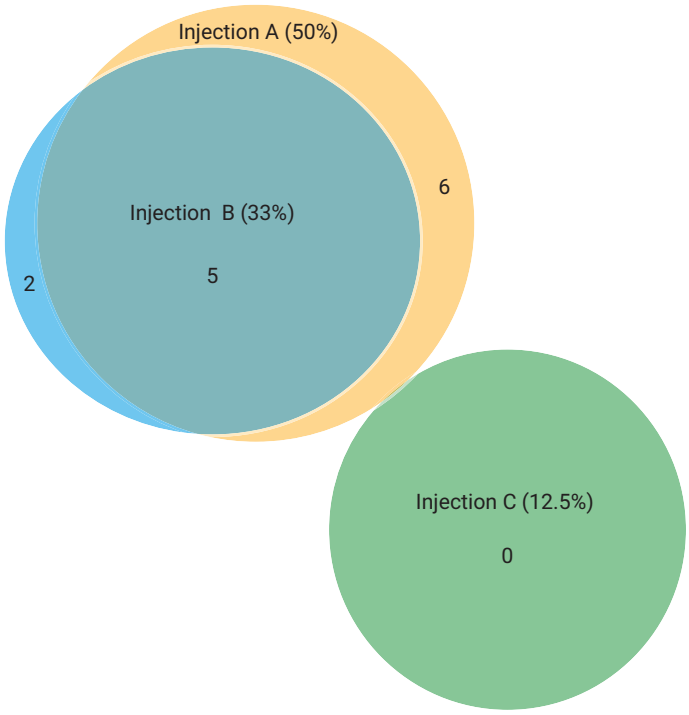

Figure 3. A summary of the number host cell proteins found per sample.

Conclusions

As expected, the largest injection amount of the AAV sample, Injection A, resulted in the detection of the largest number of proteins and obtained the highest sensitivity. Injection B was still able to detect proteins with a concentration under 1 ppm and should be enough to inject. Alternatively, the sample could be injected twice with an iterative MS/MS run. A workflow showing high analytical sensitivity using the Agilent AdvanceBio Peptide Plus column, Agilent AdvanceBio 6545XT LC/Q-TOF, and Protein Metrics Byos demonstrate high analytical sensitivity for sample-limited applications such as AAV HCP analysis.

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