Intact and Signature Peptide LCMS Bioanalysis of Three Peptide-Pegylated Lipid Conjugates from a Nanoparticle Formulation

Christian Smith[1], Ben Nie[1], Dave Beyerlein[1]

[1]BioAgilytix Labs, 9050 Camino Santa Fe, San Diego, CA 92121

BioAgilytix

POSTER # Day3-P14

BACKGROUND

Traditional bioanalysis divides potential analytes between “small molecules” which are generally analyzed by LCMS, and “large molecules” which are typically analyzed by LBA (or some form of signature fragment-based LCMS). Peptide-pegylated lipid conjugates are of an intermediate size which could be measured by either approach. In this case, all three analytes were detected as intact molecules; only two had a viable tryptic site for producing a signature peptide. We developed suitable LC/MS bioanalytical methods for analyzing the three peptide-PEGylated lipid conjugates from a nanoparticle formulation in plasma samples.

MATERIALS AND METHODS

All three analytes were obtained as individual APIs, as well as stable isotopically labeled versions labeled in the peptide portion of the molecule. Nanoparticle formulation containing all three analytes was also obtained. In brief, plasma samples were precipitated with a 1:1 mixture of acetonitrile and isopropanol. For intact analysis, the supernatant was diluted with water and injected on an Agilent PLRP-S column (300Å, 3 µm, 50x2.1mm; PN: PL1912-1301). For signature peptide analysis, the supernatant was dried down with nitrogen in a TurboVap 96, reconstituted with 5% ACN, 50 mM Tris HCl, pH 8.3, and digested with Trypsin Gold (Promega, PN: V5280) and injected on a Waters BEH C18 (130Å, 1.7 µm, 50x2.1mm; PN: 186002350).

All samples were analyzed on a Sciex 7500 mass spectrometer with ExionLC autosampler, pumps, and column oven. Representative LLOQ chromatograms can be seen below (Figure 1).

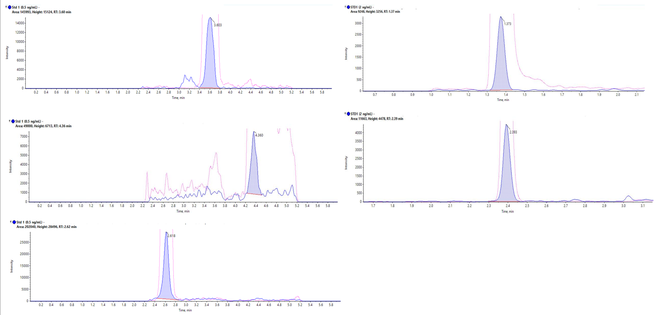

Figure 1: Representative LLOQ chromatograms for intact and tryptic digest assays. Intact assays LLOQ (left side) is 0.5 ng/mL; tryptic digest LLOQ (right side) is 2 ng/mL. For each chromatogram, the analyte trace is shown in blue with the internal standard trace overlaid in pink. The third analyte (bottom left) did not have a usable tryptic peptide for analysis.

RESULTS

Assay Performance

The tryptic peptide assays demonstrated adequate specificity, linearity, accuracy, and precision with curve ranges of approximately 2 – 2,000 ng/mL in rat, dog, and rabbit plasma/K2EDTA. The intact analysis was more sensitive, with an achievable LLOQ of 0.5 ng/mL; however, the calibration range had to be truncated due to issues with linearity.
Detecting these molecules intact on the mass spectrometer is more complex than for small molecules. Due to a combination of in-source fragmentation, multiple ionization charge states, and polydispersity of the material, the Q1 mass spectrum is quite complex (Figure 2).

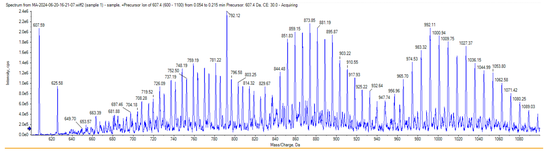

Figure 2: Precursor mass spectrum showing the variety of Q1 masses for one analyte. This results from a combination of multiple charge states and polydispersity of the PEG chain.

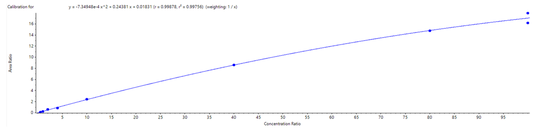

Figure 3: A calibration curve plotting area ratio vs. standard concentration demonstrating nonlinearity of response.

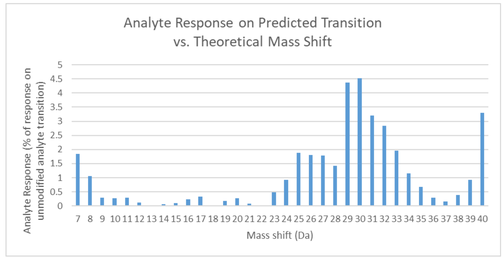

Figure 4: Mass transitions for prospective internal standards with different mass shifts were predicted, and the analyte stock was injected using each of these transitions to determine how many stable isotope labels should be added to the internal standard for minimal contribution from the analyte.

The intact assay presented additional challenges due to poor linearity (Figure 3). The internal standard initially produced for the project had a mass shift of +30 Da; however, the analyte and internal standard were each contributing signal to the other's trace, causing nonlinear response in area ratio.

Using the analyte, mass transitions for prospective internal standards were predicted and the signal contribution from the analyte on each transition was measured. A new internal standard is in the process of being synthesized based on this data, which will be used for validation and sample analysis of the intact assay.

Accuracy and Precision of the Tryptic Assay

While validation work has not been conducted due to the new internal standard synthesis being in progress, unregulated sample analysis was conducted for the tryptic assay with a truncated range. The curve and QCs were prepared from nanoparticle formulation, making it impossible to set the curve ranges exactly equal for each analyte, but the ranges were approximately 2-200 ng/mL for one analyte, and 3-300 ng/mL for the other. Quality control sample data is presented below.

Analyte 1			
Concentrations (ng/mL)			
Nominal	**5.60**	**56.0**	**140**
Run 1	4.45	47.9	139
	4.69	47.8	147
	4.51	46.4	143
Run 2	5.38	52.1	137
	5.59	55.7	141
	5.42	55.2	138
Average	5.01	50.9	141
%CV	10.2	7.96	2.63
%DEV	-10.5	-9.11	0.714

Analyte 2			
Concentrations (ng/mL)			
Nominal	**9.04**	**90.4**	**226**
Run 1	7.36	80.9	226
	7.63	79.1	230
	7.90	77.8	224
Run 2	8.00	85.5	229
	8.90	88.3	227
	8.33	85.1	227
Average	8.02	82.8	227
%CV	6.77	4.98	0.941
%DEV	-11.3	-8.43	0.516

NOVELTY ASPECTS AND CONCLUSIONS

Herein, we present development of robust methods to detect three Peptide-Pegylated lipid Conjugates by Intact and Signature Peptide LCMS to support preclinical studies. Two of the analytes were analyzed by a signature peptide approach using trypsin, while all three were detected as intact molecules from a simple protein precipitation.

- In addition to challenges with ionization causing poor sensitivity, intact LCMS is difficult for large molecules since they are frequently not monodispersed. While these analytes had sufficient sensitivity to be detected intact at biologically relevant concentrations, variations in the population were encountered which caused each analyte to be found at a variety of masses.
- For LCMS analysis it is necessary that the lot of reference standard should be matched to the lot which was used to dose the samples, and if possible, multiple lots of reference standard should be screened to ensure the chosen ions will remain sufficiently abundant for assay performance to be maintained. For both scientific and logistical reasons, it is preferable to prepare calibration curve and quality control samples from the formulated nanoparticle. This approach puts restrictions on the ability to define a precise curve range for multi-analyte assays.
- Finally, internal standards merit special consideration for this type of analysis. Typically, stable isotopically labeled internal standards are used for small molecule LCMS, while no internal standard is used for LBA. For signature fragment LCMS analysis, producing an isotopically-labeled large molecule is often infeasible, and an isotopically labeled fragment is used instead. In this case, stable isotopically-labeled versions of each analyte were obtained; however, due to the polydispersed nature of the pegylated intact molecules, some amount of “cross-talk” between analyte and IS traces was difficult to avoid. However, mass transitions of hypothetical internal standards can be predicted from behavior of the unlabeled analytes, and tested on reference standard to choose how many isotopic labels should be used to minimize this interference.