Quantitative Analysis of P-15 Peptide in Hydroxyapatite Matrix using LC/MS/MS

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Introduction

P-15 is a synthetic, 15-amino-acid residue, identical to the sequence (766)GTPGPQGIAGQRGVV(780) found in the a1(I) chain of type I collagen. Hydroxyapatite (HA) is the mineral component of bone and consists primarily of calcium and phosphorus. P-15 bone graft substitute (P15-BGS) is a combination of the mineral component of bone and the P-15 peptide. It is thought to enhance cell attachment and extracellular matrix and factor production, resulting in the formation of bone. P-15 may also encourage the integration of artificial bone with natural bone. It is currently being tested in clinical trials to aid bone formation during spinal fusion surgery[1].

Extraction of the peptide from the bone mineral matrix involves addition of 1M HCl. The acid dissolves the HA releasing the peptide. The resulting solution has very high levels of phosphates, calcium and chloride.

We have developed a quick and simple method involving online sample enrichment/cleanup followed by analytical separation of the peptide[2]. We can detect the peptide down to attamole levels on column. In the past, it was possible to achieve this level of sensitivity for quantitative proteomics with nanoflow LC. In this work we demonstrate attamole level sensitivity at regular LC flows of 0.3 mL/min. This is possible because of the high ion sampling efficiency of the 6490 QQQ.

Experimental

Agilent 1290 Infinity UHPLC:

Analytical Column: Agilent Rapid Resolution High Definition (RRHD) Zorbax Eclipse Plus C18, 2.1x150mm, 1.8 µm Mobile Phase: A=Water+0.1% Formic acid, B=ACN+0.1%Formic acid; Injection volume: 20 µL, Column flow: 0.3 mL/min, Column temperature: 50°C,Gradient: 3% B for 2.5 minutes, 3% to 45 % B in 2 minutes, Flush with 95% B for 2 minutes, Equilibrate back to 3%B for 2 minutes. Run time: 9 minutes, Loading Column: 30 mm C18 Cartridge. Loading conditions: 3% B for 3 minutes, flush with 95% B for 2 minutes, equilibrate back to 3%B for 3 minutes.

Agilent 6490 Triple Quadrupole MS:

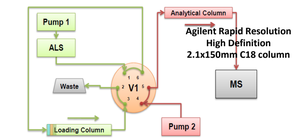
MRM Transitions: 697.4→856.5, 697.4→686.4, Dwell time:100 ms, Resolution: MS1/MS2: unit/unit (Q1: 0.7m/z/Q2: 0.7 m/z),Polarity: Positive

Parameters: Drying gas temperature: 200 °C, Drying gas flow: 12 L/min, Sheath gas temperature: 300°C, Sheath gas flow:12 L/min, Nebulizer pressure: 45 psig, Nozzle Voltage: 500 V, Capillary voltage: 4000 V

Experimental

2D System: Enrichment – Interval 1

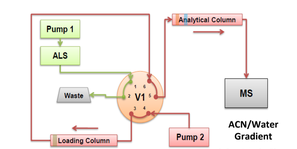
Valve position 1 - Loading Column: Injected with complex sample, elutes un-retained compounds to waste
Analytical Column: Equilibrated, ready for analysis



30mm C18 Cartridge, 20 µL Injection Volume

2D System: Enrichment – Interval 2

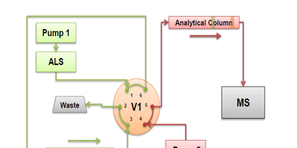
Valve position 2 - Loading Column elutes all trapped compounds into Analytical Column



ACN/Water Gradient
0.3 mL/min Flow

2D System: Enrichment – Interval 3

Valve position 1 - Loading Column: Equilibrated and loaded with next sample
Analytical Column: Elutes all compounds to MS and is equilibrated ready for next analysis



Flush with 95% organic and equilibrate back

Results and Discussion

Figure A 710 zeptomoles/µL Standard solution, ~14 attamoles on column

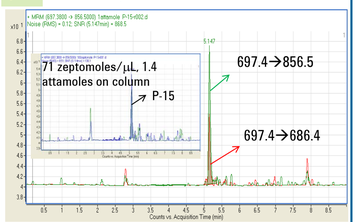


Figure B 7 attamoles/µL HA solution and 70 attamoles/ µL HA solution Chromatograms Ssuperimposed

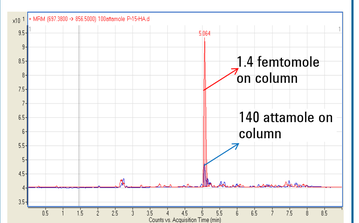


Figure C P-15 in actual Bone Graft Sample

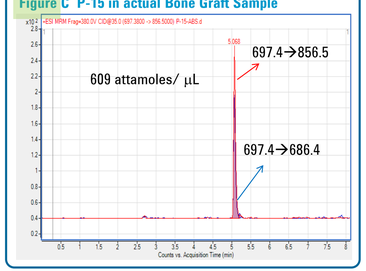
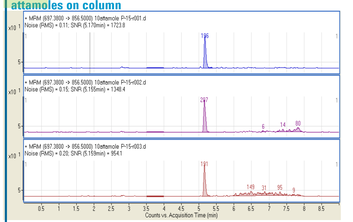


Figure D Reproducibility of three injections: 4% RSD, 140 attamoles on column



Standard Chromatograms

P-15 standard solutions were prepared in the concentration range of 70 zeptomole/µL to 0.7picomole/µL. With clean standard and no matrix, it is possible to detect the peptide at the 70 zeptomole/µL level. With a 20 µL injection, this amounts to 1.4 attamoles injected on column. The superimposed chromatograms for the two transitions monitored for the 1.4 attamole on column is shown in the insert of Figure A. Figure A shows the peaks corresponding to the 14 attamole on column.

Reproducibility for three injection of the 7 attamoles/µL level is in Figure D. RSD% was found to be 4%.

Figure B shows the response for the peptide spiked into the HA matrix. The superimposed chromatograms show the peak for the 7 attamoles/µL level and the 70 attamole/µL level .

Figure C shows P-15 in the actual bone graft sample at a level of 609 attamoles/µL. Quantitation for the sample was done by building a calibration curve by spiking the peptide into the HA matrix.

Calibration Curves

The calibration curves in the range of 70 zeptomoles/µL to 0.7 picomoles/µL are shown in Figure E and Figure F. Correlation Coefficients (R^2) were found to be 0.999 and 0.996 respectively. Area RSD% ranged from 1 to 11%.

Calibration curve for P-15 in HA matrix is shown in Figure G. Correlation Coefficient (R^2) was found to be 0.998. This curve was used to quantitate the actual bone graft sample.

Results and Discussion

Figure E Calibration Curve 70 Zeptomoles/µL to 7 Femtomoles/µL

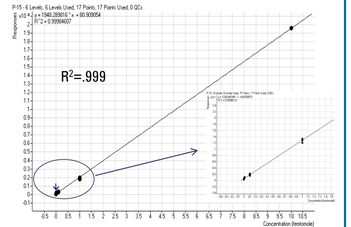


Figure F Calibration Curve 70 Zeptomoles/µL to 0.7 Picomoles/µL

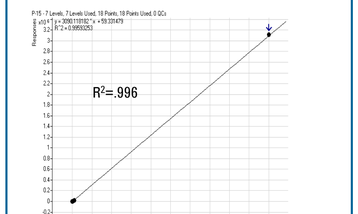
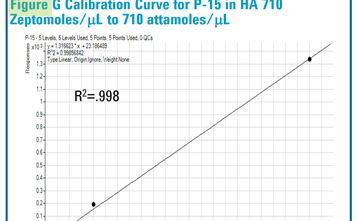


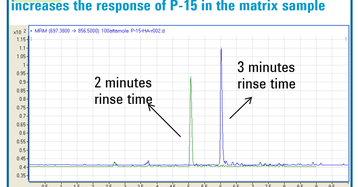
Figure G Calibration Curve for P-15 in HA 710 Zeptomoles/µL to 710 attamoles/µL



Future Work

We are currently investigating the duration of the sample loading/rinse step to clean up the matrix. Initial studies indicate that increasing the rinse time from 2 to 3 minutes increases the response as shown in Figure H below.

Figure H Increasing the rinse time from 2 to 3 minutes increases the response of P-15 in the matrix sample



Conclusions

- Online sample cleanup works well for rinsing the very high concentration/complex bone mineral matrix from the peptide
- The increased sensitivity of the 6490 with the dual ion funnel allows us to achieve attamole levels under normal UHPLC flow conditions.
- Sensitivity and linearity of the method is demonstrated from zeptomole to picomole levels.
- LOD for the P-15 peptide standard is 70 zeptomoles/µL.
- LOD for the P-15 peptide in HA matrix is about 7 attamoles/µL

References

Reference 1. Liu, Q., Limthongkul, W., Sidhu, G., Zhang, J., Vaccaro, A., Shenck, R., Hickok, N., Shapiro, I. and Freeman, T. (April 2012), Covalent attachment of P15 peptide to titanium surfaces enhances cell attachment, spreading, and osteogenic gene expression. J. Orthop. Res.. doi: 10.1002/jor.22116 and references therein.

Reference 2. The authors are not aware of any other published LC/MS method for the analysis of this peptide.