LC-MS/MS QUANTIFICATION OF INTACT INSULIN-LIKE GROWTH FACTOR-1 FOR CLINICAL RESEARCH

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INTRODUCTION

Insulin like Growth Factor I (IGF-I) is a 7.6kDa protein, with 3 internal di-sulphide bonds, which plays a significant role in mediating the effects of Growth Hormone (GH). IGF-I has been used as a supplementary or surrogate marker for GH. In recent years, use of IGF-I as a marker for GH related doping has also been reported. IGF-I is found in circulation throughout the body as a complex bound to one of 6 binding proteins, the most significant of which is IGF binding protein 3 (IGF-BP3).

Historically, immunoassays have been used for quantification of IGF-I from human biological matrices. In recent years, LC-MS based approaches have been reported, which typically utilize digestion and quantification via the surrogate peptide approach, often on a tandem quadrupole instrument. Although the surrogate peptide approach for quantification of proteins is widely accepted, it is not necessary for a small protein such as IGF-1.

Affinity enrichment approaches[2] have also been reported to clean up the samples before quantification of intact IGF-I using a nano-UPLC HRMS system[3]. These approaches require significant time and resources adding unnecessary cost and complexity to the analysis.

Here, we present an LC-MS/MS method for direct quantification of intact IGF-I for clinical research. This work applies a simple SPE clean up, tandem quadrupole MS, and analytical scale UHPLC to achieve industry leading lower limits of quantification in the single digit ng/mL range.

Amino acid Sequence:

GPETLCGAEL VDALQFVCGD RGFYFNKPTG YGSSSRRAPQ TGIVDECCFR SCDLRRLEMY CAPLKPAKSA

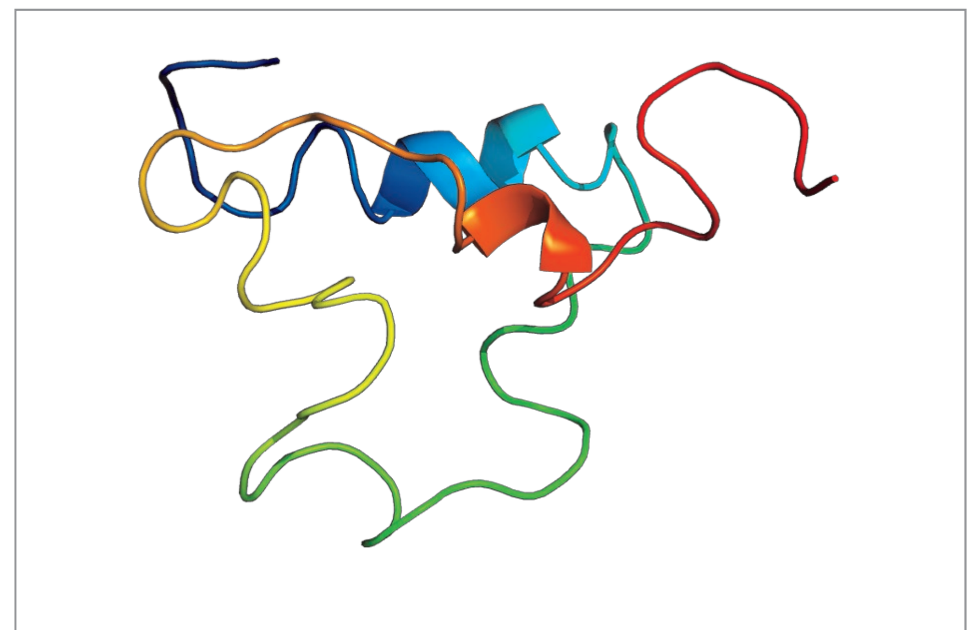
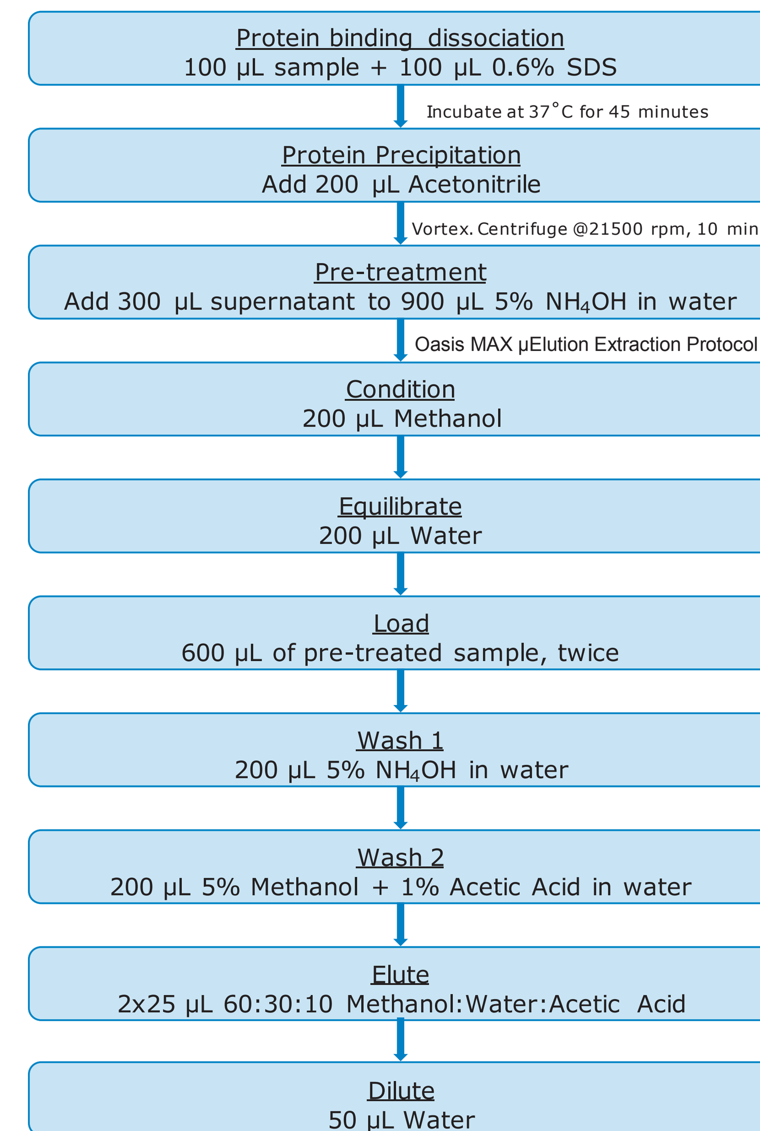


Figure 1. Intact IGF-I structure. [1]

METHODS

Sample Extraction



LC Method

Instrument: Waters ACQUITY UPLC I-Class System
Column: CORTECS C18+, 90 Å, 1.6 µm, 2.1 mm X 50 mm
Column temperature: 60°C; Sample temperature: 5°C
LC Gradient:

Time (mins)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.400	95	5	6
2.5	0.400	70	30	6
3.5	0.400	50	50	6
3.6	0.400	5	95	6
4.0	0.400	5	95	6
4.1	0.400	95	5	6
5.0	0.400	95	5	6

MS Method

Instrument: Waters Xevo TQ-XS tandem quadrupole mass spectrometer

Tune Page parameters

Parameter	Value
Capillary Voltage (kV)	1
Cone Voltage (V)	30
Desolvation Temp (˚C)	500
Desolvation (L/Hr)	800
Cone (L/Hr)	150
LM1 & LM2	2.5
HM1 & HM2	14.0

Transitions

Precursor (m/z)	Product (m/z)	CE (eV)	Cone (V)
1093 (+7)	1196.4	35	30
1093 (+7)	473.4	40	30
956.4 (+8)	1196.4	30	30
956.4 (+8)	1175.2	30	30
956.4 (+8)	473.4	30	30

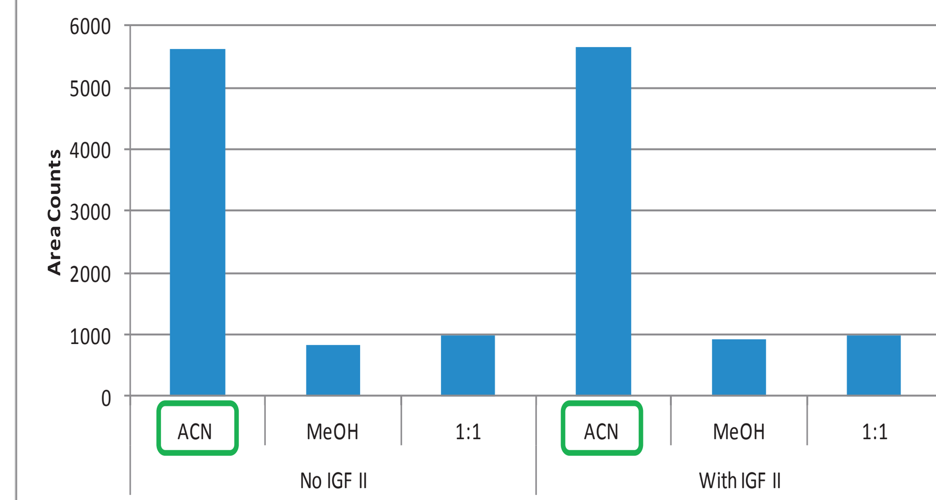


Figure 2. Evaluation of different solvents used for protein precipitation. ACN gave the best results. IGF-II did not interfere with extraction.

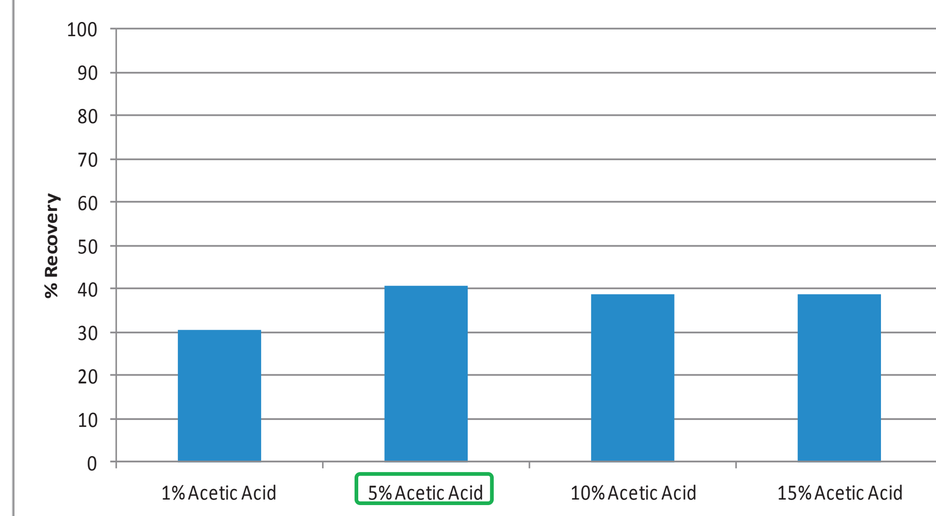


Figure 3. Evaluation of different % acid to disrupt protein binding. 5% Acetic Acid gave the highest recovery of ≈40%

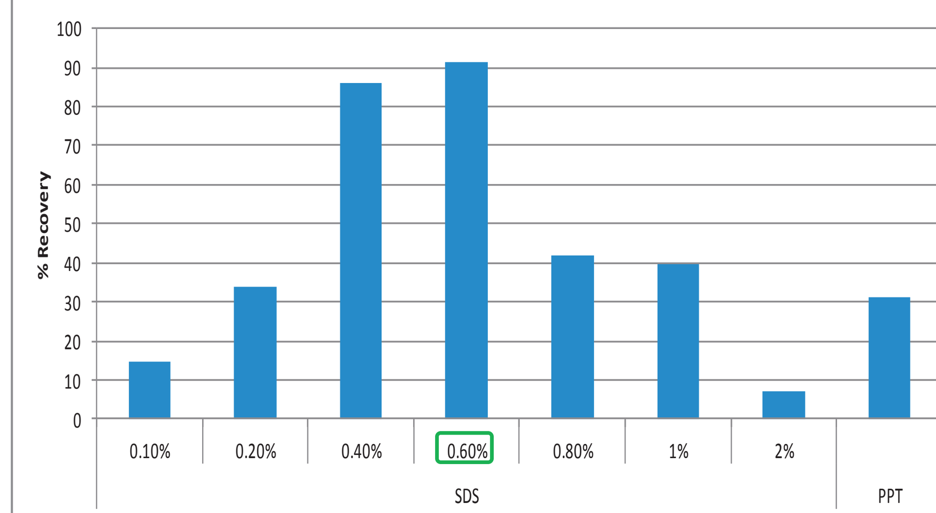


Figure 4. Evaluation of different % SDS to disrupt protein binding. 0.60% SDS gave the highest recovery at ≈90%

RESULTS & DISCUSSION

Quantifying a 7.6 kDa protein like IGF-I intact on a tandem quadrupole instrument requires a very meticulous and methodical approach to every step in the workflow. All aspects, beginning from evaluating the best reconstitution solvent for lyophilized standards, all the way through to the sample extraction and LC-MS method parameters need careful scrutiny.

Sample extraction

Circulating IGF-I binds very strongly to its binding partner, Insulin-like Growth Factor Binding Protein (IGFBP). In order to accurately quantify IGF-I levels, it is imperative to disrupt this protein binding. IGF-II, which also binds IGFBP strongly, was added in excess during extraction to prevent IGF-I—IGFBP complex form forming post disruption. ACN, MeOH and a 1:1 ratio of ACN:MeOH were evaluated. ACN gave the best results (Figure 2). The presence of IGF-II did not have any effect on extraction of IGF-I using ACN. To disrupt protein binding, different types of acids, and different concentrations of each acid were assessed. Best results were obtained using 5% Acetic acid (Figure 3). The recoveries using 5% Acetic acid were around 40%, indicating that the IGF-I—IGFBP complex was not completely disrupted. Other reagents used to disrupt protein binding, like $ZnSO_4$, Guanidine and SDS were also assessed. 0.60% SDS achieved the highest recovery of >90% (Figure 4).

Calibration Curve & QC samples

Since IGF-I is an endogenous biomarker, mouse plasma was used as a surrogate matrix. IGF-I was spiked in mouse plasma in the range of 5 - 1000 ng/mL. The calibration curve was linear from 5-1000 ng/mL with r^2 >0.99 using 1/x weighting for at least 2 MRM transitions monitored (Figure 5). All points on the curve, including the LLOQ (5 ng/mL) were robust and reproducible (Figure 6). A mix of mouse and human plasma samples were used to make up QC samples. For the LQC, mouse plasma was spiked at 25 ng/mL. For MQC, unspiked human serum, which had endogenous levels of IGF-I at around 100 ng/mL was used. HQC was prepared by spiking an additional 500 ng/mL into the human serum, to give a final concentration of approximately 600 ng/mL (Figure 7). The % CV and % bias across all levels of QC were <10% (Table 1), which are well within the bioanalytical validation criteria. Acceptable % bias for MQC and HQC which are made in human serum proves that a surrogate mouse curve can be used to quantify human serum samples.

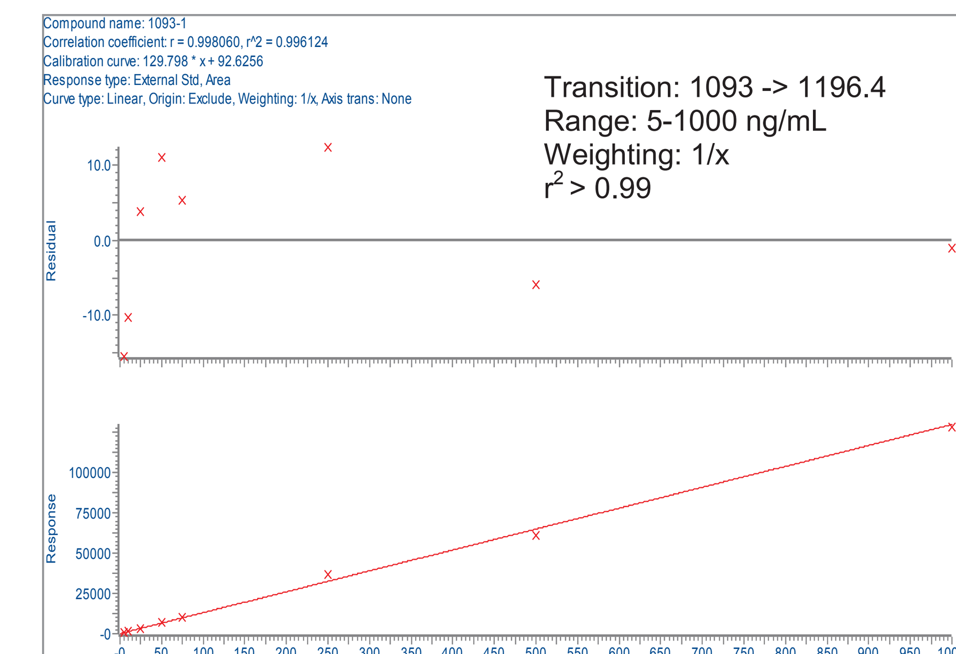


Figure 5. Representative calibration curve and residual plot. Curve is linear form 5-1000 ng/mL. % Bias for all points <15%

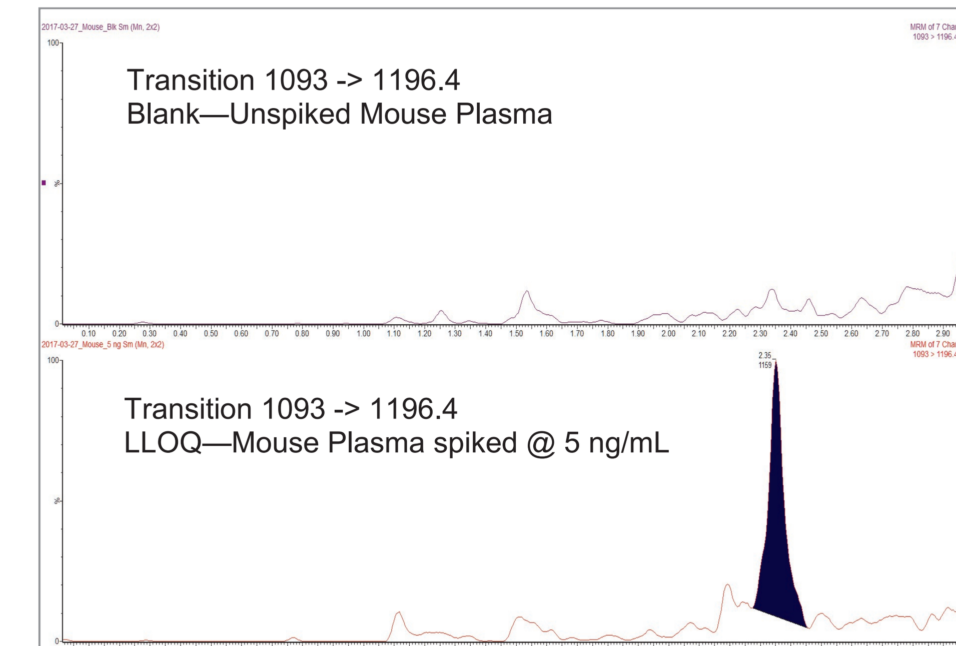


Figure 6. Representative Chromatograms for Blank and LLOQ samples
Chromatograms for Blank & LLOQ samples for transition 1093->1196.4

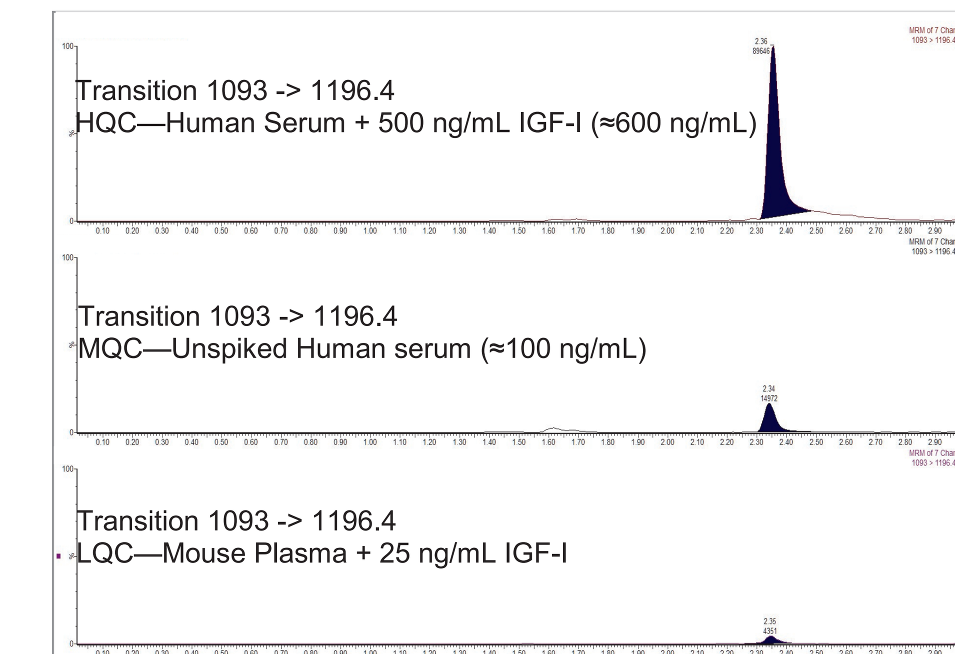


Figure 7. Representative chromatograms for QC's
Chromatograms for QC levels for transition 1093->1196.4

	LQC	MQC	HQC
Replicate 1	23.8	110.7	680.5
Replicate 2	25.1	99.3	620.7
Replicate 3	24.4	91.1	594.7
Expected	25	100	600
Observed	24.43	100.37	631.97
Std Deviation	0.65	9.84	44.00
% CV	**2.66**	**9.81**	**6.96**
% Bias	**2.32**	**-0.37**	**-5.06**

Table 1. Representative QC Statistics table
% CV & % Bias for all QC levels <10%

CONCLUSIONS

- **A simple sample preparation approach which included, protein precipitation, denaturation and mixed-mode SPE, achieved high IGF-1 recovery while providing selectivity.**
- **No complex sample extraction procedures like protein digestion or affinity chromatography were used.**
- **This method can accurately quantify intact IGF-I from human serum in the range of 5-1000 ng/mL using analytical scale LC/MS.**
- **The method is robust and reproducible with % CVs and bias <15%, easily achieving recommended criteria for bioanalytical quantification.**
- **The analytical sensitivity (5 ng/mL), linear dynamic range, and excellent reproducibility of the method described reliably measures low endogenous and levels of IGF-1.**

REFERENCES

1. https://commons.wikimedia.org/wiki/File:Protein_IGF1_PDB_1bqt.png#/media/
2. Niederkofler EE, Phillips DA, Krastins B, Kulasingam V, Kiernan UA, et al. (2013) Targeted Selected Reaction Monitoring Mass Spectrometric Immunoassay for Insulin-like Growth Factor 1. PLoS ONE 8(11): e81125. doi:10.1371/journal.pone.0081125
3. Filipe Lopes, David A. Cowan, Mario Thevis, Andreas Thomas and Mark C. Parkin, (2014) Quantification of intact human insulin-like growth factor-I in serum by nano-ultrahigh-performance liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom., 28, 1426–1432

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