Can Human Therapeutic Antibody (Humira) be Analyzed by LC-MS/MS in Human Plasma Without Immuno-Affinity Enrichment?

Luca Genovesi, Jean-Nicholas Mess and Fabio Garofolo*

ALTASCIENCES

Altasciences, 575 Armand-Frappier, Laval, Québec, H7V 4B3, Canada
Altasciences.com | contact@altasciences.com

OVERVIEW

Purpose

- Quantify Humira (Adalimumab) by LC-MS, using an immuno-affinity enrichment-free method suitable for comparability studies of biosimilar and innovator biologics.

Method

- Human plasma samples were spiked with Humira.
- Proteins were digested with trypsin, pepsin or chemically cleaved under various conditions.
- Extracts were analyzed by LC-MS on an API5000 operated in ESI+ mode or a TripleTOF™ 5600.

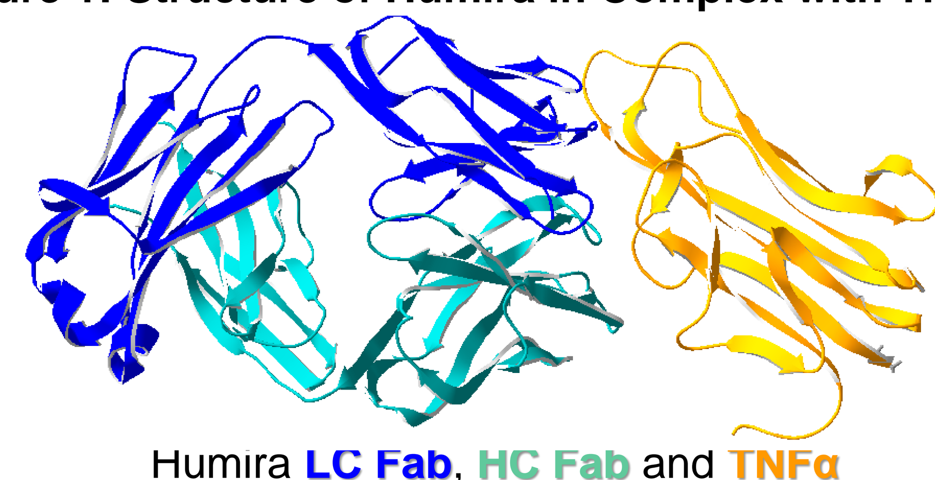
Results

- Immuno-Affinity free: not suitable for clinical studies due to a peptide interference in the blanks; works for pre-clinical samples.
- TNFα purification: a linear, robust and reproducible assay, suitable for biosimilar/bionnovator analysis; performances comparable to current ELISA assay.

INTRODUCTION

Protein therapeutics are highly successful in the clinic, currently enjoying unprecedented recognition of their potential. More than 100 therapeutic proteins are currently approved for clinical use in the European Union and the USA. A biosimilar, according to the FDA definition, is a subsequent version of an innovator biologic with no meaningful differences in safety, purity and potency. Given the complexity of the biosimilar production process, structural heterogeneities may arise that render ligand binding assays non-functional. Immuno-affinity enrichment-free approaches like LC-MS are generally not affected by these structural changes, therefore are fit for purpose in generating a universal assay. In this study, we present the challenges observed in the development of an immuno-affinity enrichment-free LC-MS assay for the quantification of Humira (Adalimumab) in human plasma.

Figure 1: Structure of Humira in Complex with TNFα



Humira **LC Fab**, **HC Fab** and **TNFα**

METHODS Enrichment Free Approach

Tryptic Peptide Selection

Humira public sequence has been in silico digested with trypsin. Peptides found to be unique to Humira in the human proteome are indicated in blue in **Figure 2**.

Figure 2: Humira Protein Sequence with Three Humira Specific Tryptic Peptides Highlighted

Light chain:

DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYAASTLQSGVPSR**FSGS**
GSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA
SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGEC

Heavy Chain:

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGK**GLEWVSAITWNSGHIDYADSVE**
GRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAK**VSYLSTASSLDYWGQGTLVTVSSASTK**GPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
SLGTQTYICNVNHKPSNTKVDKKVEPKSC

Sample preparation

- Proteins were denatured with 8M urea, reduced with TCEP and alkylated with IAM in the dark.
- Digestion was performed with low grade bovine trypsin.
- Samples were desalted on OASIS HLB 60mg.

Chromatography

- Agilent Technologies Series 1100
- XBridge Peptide BEH C18 (50 x 2.1mm, 3.5µ)
- Gradient of 0.1% HCOOH in H_2O and MeOH

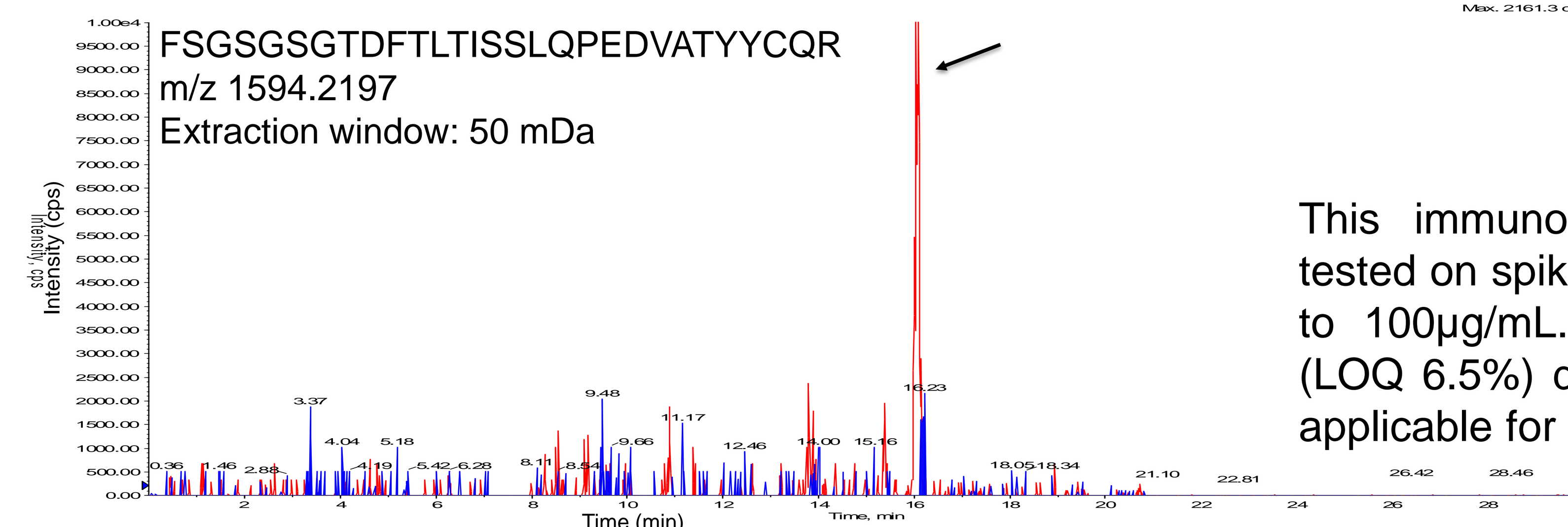
Detection

- AB SCIEX TripleTOF™5600 or API5000

RESULTS Enrichment Free Approach

All of Humira specific peptides identified (**Figure 2**) have an m/z for $[M+2H]^{2+}$ out the calibration range of the API5000, exact mass TOF-MS was the only way to analyze them. Among the 3 unique peptides, FSGS had the best intensity, but its sensitivity was inadequate to reach the LOQ needed (**Figure 3**).

Figure 3: Extracted Human Plasma Blank (blue) and 100 µg/mL Humira (red) for Peptide FSGS Analyzed by TOF-MS

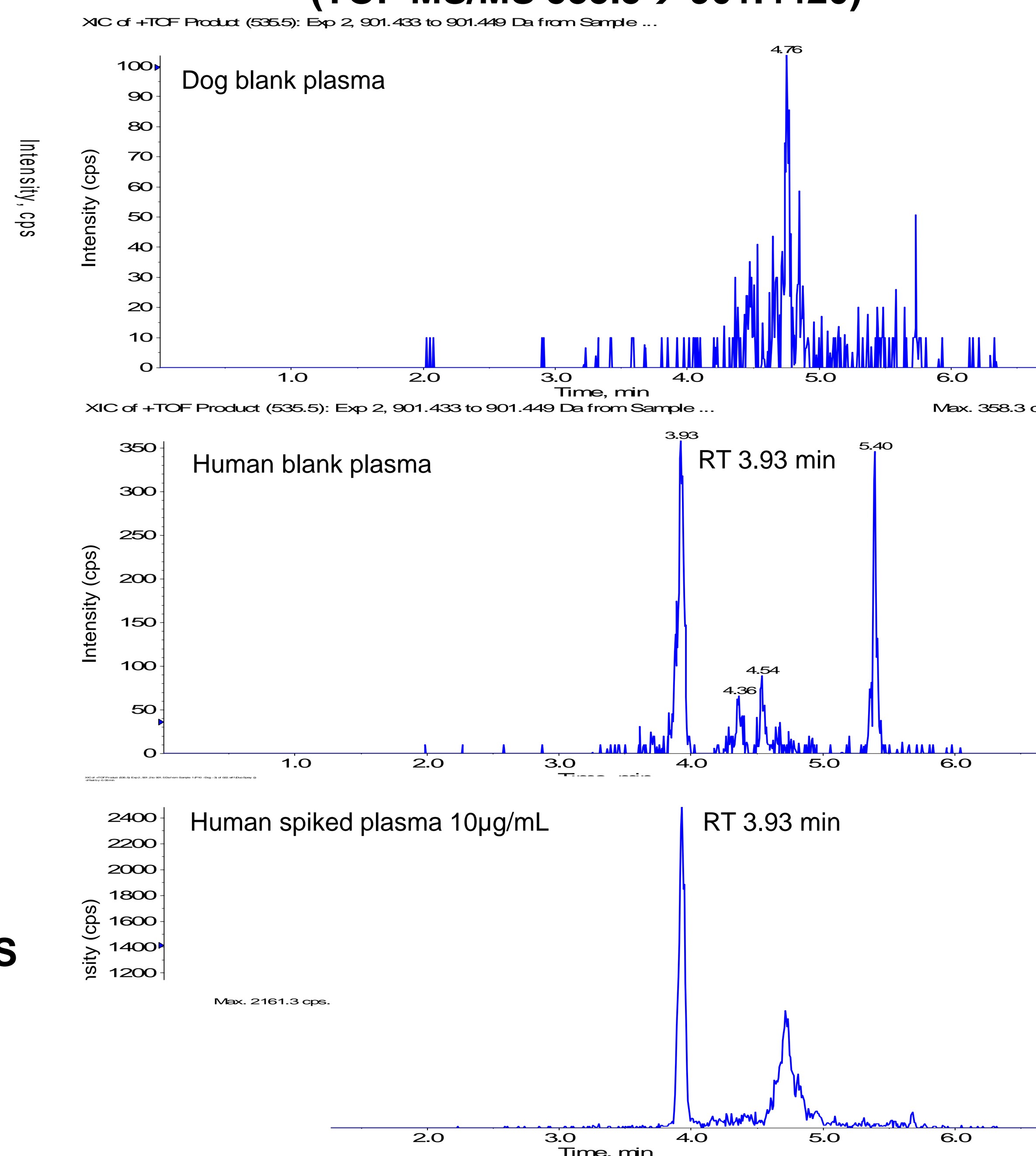


Alternative Peptide Selection

Peptide APYTFGQGTK has emerged as an alternative signature peptide due to its high yield and because it was found to be shared only with one protein from the human proteome, the anti-tetanus toxoid immunoglobulin.

Unfortunately, an interfering peak was constantly observed in digested human plasma. Blank plasma coming from different species (dog, rat and mouse) were tested and those samples resulted clean of interferences (**Figure 4**). Additionally, TOF MS/MS scan analysis revealed that the interfering peptide had the same exact mass and fragmentation pattern as APYTFGQGTK. Therefore, this peptide was proven endogenous as per BlastP alignment and the approach not feasible.

Figure 4: APYTFGQGTK in dog and human blank plasma, and in Humira spiked human plasma (TOF MS/MS 535.5→ 901.4420)



This immuno-affinity enrichment-free method was then tested on spiked dog plasma samples for a range from 1.00 to 100µg/mL. Linearity (0.999) and reproducibility CV% (LOQ 6.5%) demonstrated that this method is theoretically applicable for pre-clinical samples.

Alternative Digestion Procedure

Pepsin and NTCB in silico digestion provided some unique peptide, however they lacked in specificity and reproducibility when tested experimentally.

Derivatization of Lysine residue to make trypsin work as Arg-C has been demonstrated successful in buffer with Acetate NHS as derivatizing agent. However, in plasma samples, the original tryptic peptide was still the predominant species, indicating that the Lysine derivatization was inefficient.

METHODS Affinity Enrichment Approach

By selecting the natural target of Humira as a bait for affinity capture, all of the possible heterogeneity between the biosimilar and the innovator should be cancelled, because the bioactivity, as per definition, should remain identical.

Sample preparation

- TNF alpha was biotinylated with EZ-Link Sulfo-NHS-Biotin
- TNF alpha was then coupled with chemicell magnetic beads with a ratio of 1:10 TNF-beads slurry
- Plasma was incubated for 2 hours with 50µL of beads, then digestion was performed as previously described.

Chromatography

- Agilent Technologies Series 1100 pumps and autosampler
- XBridge Peptide BEH C18 (50 x 2.1mm, 3.5µ)
- Gradient of 0.1% HCOOH in H_2O and MeOH

Detection

- API5000 operated in ESI(+) mode
- Signature peptide APYTFGQGTK: 535.5→901.4
- Analog Peptide (IS): 549.8→766.6

RESULTS Affinity Enrichment Approach

This method was tested with Humira spiked samples from 0.10 to 10.00 µg/mL. Good results in terms of linearity (0.999), specificity and reproducibility (CV%=5.2) were achieved with no observed carryover (**Figures 5 and 6**). The superior sensitivity obtained, combined with the great specificity, makes this method suitable to further decrease the LLOQ in the range of 10 ng/mL, competing with best performing ELISA methods, but having a greater dynamic range. The analogue peptide used as internal standard showed good performances in tracking injections and demonstrating the solidity of the extraction and chromatography.

Figure 5: Blank Extracted Plasma (Blue), 0.1µg/mL Spiked Humira (red) and IS (black) chromatograms

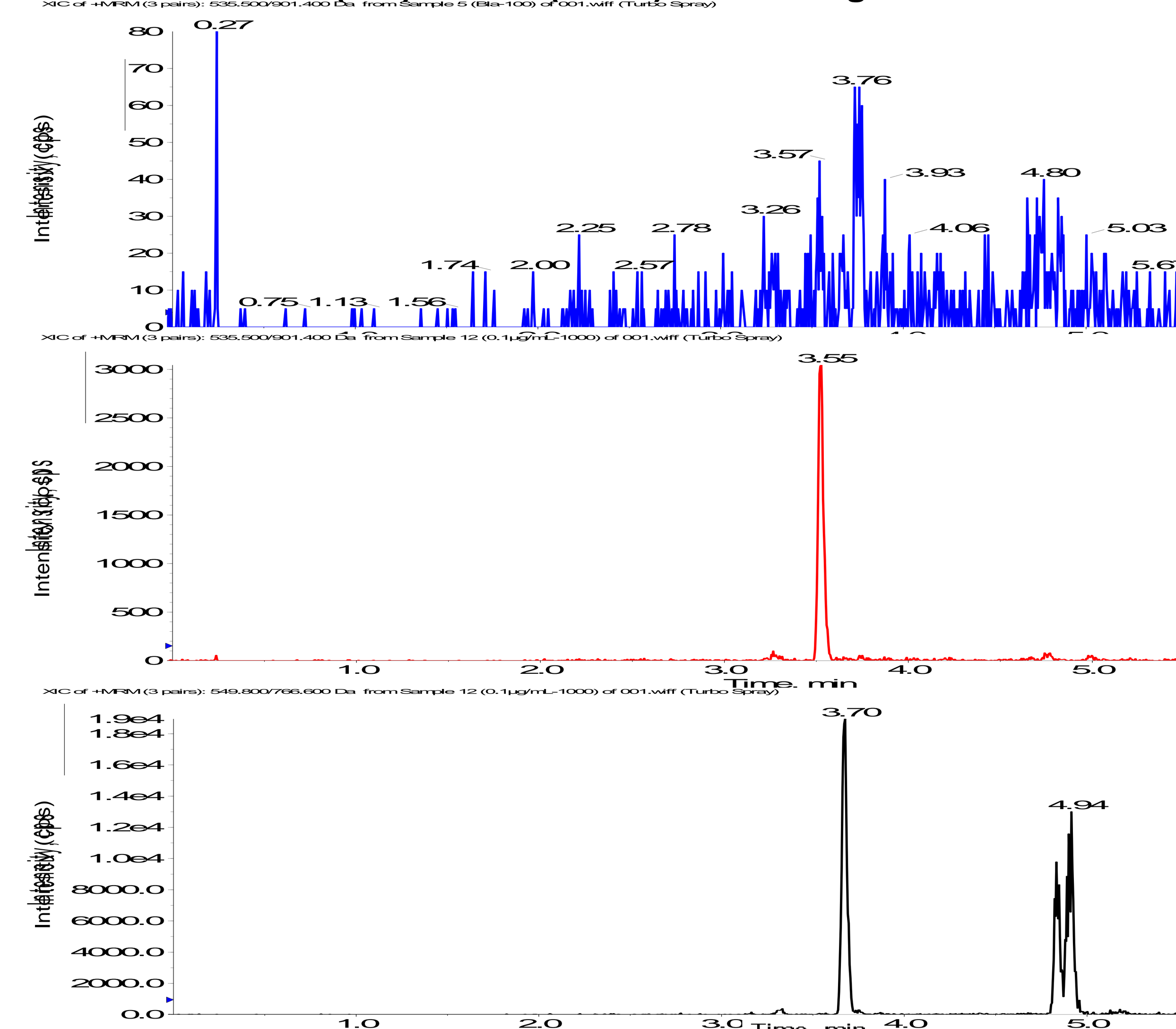
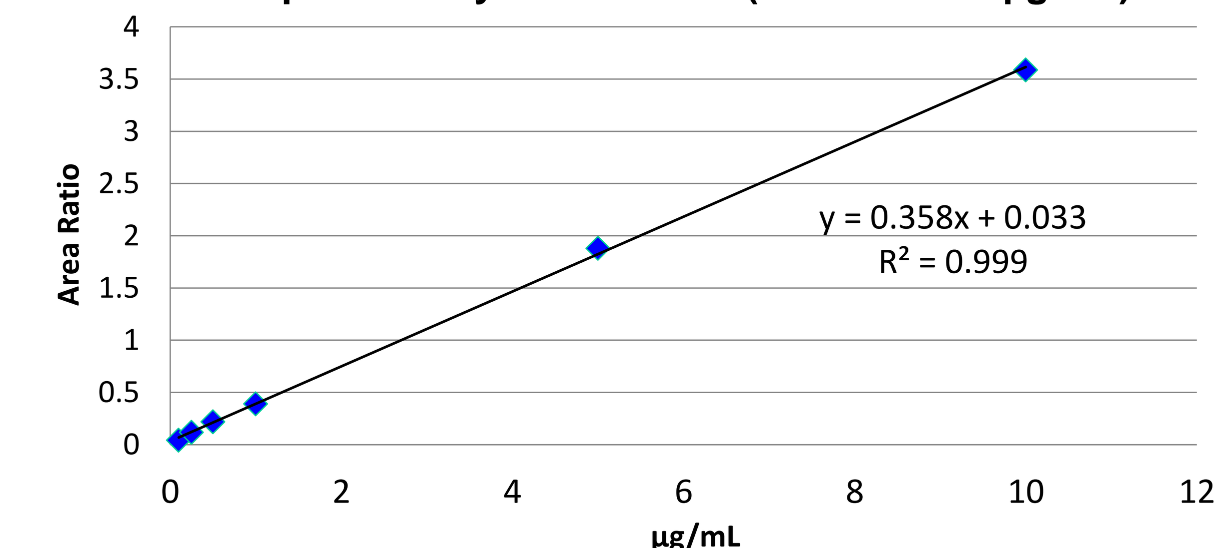


Figure 6: Calibration Curve of Humira in Human Plasma Using TNF-alpha Affinity Enrichment (0.10 to 10.00 µg/mL)



CONCLUSION

The development of an immuno-affinity enrichment-free assay for Humira in human plasma presents several challenges due to the nature of Humira, a human antibody therapeutic.

The TNFalpha-affinity enrichment quantification method proved to be a proficient way to overcome the obstacles all at once, allowing the final method to cover a dynamic range of 3 orders of magnitude. The use of the biological target for the purification makes this assay robust, reliable and universal for all possible biosimilar/bioinnovator products of Humira.

LEARN MORE ABOUT OUR BIOANALYSIS AND RESEARCH SERVICES

* Presently at Angelini Pharma