HYBRID LC-MS/MS FOR QUANTIFICATION OF INFLIXIMAB IN CROHN'S DISEASE PATIENT SAMPLES: DOES IT ADD VALUE?

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INTRODUCTION

Therapeutic drug monitoring (TDM) of tumor necrosis factor alpha (TNF-α) inhibitors, such as infliximab (IFX), plays an important role in optimization of therapy and understanding of non-response (1yr or 2yr) which is not uncommon. Harmonization towards a standardized approach is being driven by variability between commercially available ELISA kits. Free drug is measured as an indicator of active drug, however decision making based on TDM is complicated by different therapeutic thresholds. LC-MS/MS has many redeeming benefits compared to ELISA, which is recognized as relatively simple and inexpensive. Direct digestion and quantification using selected surrogate peptides can measure total drug. Concerns with this approach arise when ambiguity in correlating multiple surrogate peptides with ELISA is observed. Alternatively, free drug can be measured using a hybrid LC-MS/MS approach employing a highly specific TNF-α antigen capture reagent. This work describes a new unpublished dataset acquired using human serum Crohn's Disease (CD) patient samples and hybrid LC-MS/MS (TNF-α capture reagent) for comparison with existing LC-MS/MS and ELISA datasets. This clinical research highlights the benefits, challenges and applicability of these techniques for standardized TDM.

METHODS

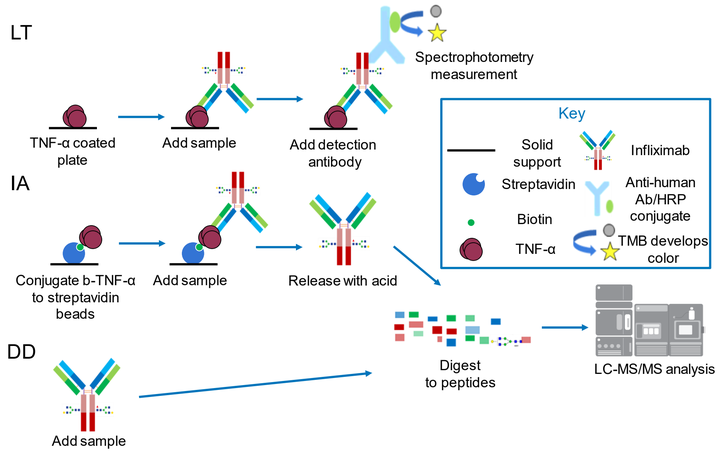
Trough samples collected from CD patients on maintenance infliximab were analyzed by automated LISA TRACKER ELISA (Theradiag, France), direct digestion LC-MS/MS, and hybrid LC-MS/MS, also known as immunoaffinity (IA) LC-MS/MS (Table 1). Direct digestion LC-MS/MS analysis was performed using 25 µL of serum, diluted with digest buffer prior to digestion. Hybrid LC-MS/MS was performed using 5 µL of serum, and affinity capture with the target antigen, TNF-α, which was biotinylated and bound to commercially available streptavidin magnetic beads (Figure 1). All LC-MS/MS samples were digested using ProteinWorks eXpress Digest Kit's standardized protocol. LC-MS/MS quantification of the resulting signature tryptic peptides was performed using ACQUITY I-Class UPLC PLUS , coupled to a Waters Xevo TQ-XS tandem quadrupole MS (ESI+). Chromatographic separation was achieved using a Peptide BEH C_{18}, 300Å, 1.7 µm, 2.1 x 150 mm column, at a flow rate of 0.3 mL/min using a linear gradient with 0.1% formic acid in water and acetonitrile.

Assay Characteristics			
Assay Name	**Assay Type**	**IFX Capture**	**Measurement Range (µg/mL)**
LISA TRACKER (LT)	ELISA	TNF-α	0.3 – 16.0
Hybrid (IA)	IA-LC-MS/MS	TNF-α	0.1 – 50.0
Direct Digestion (DD)	LC-MS/MS	None	1.0 – 100.0

Table 1. Characteristics of infliximab assays.

Figure 1. Principles of infliximab quantification assays.

- *(LT) Samples are added to TNF-α coated microplates, infliximab is purified from the sample, and detected via spectrophotometry*
- *(IA) Samples are added to streptavidin coated magnetic beads which are conjugated to TNF-α. Infliximab is purified from the sample, digested to peptides, and detected via LC-MS/MS analysis*
- *(DD) Samples are digested to peptides directly from serum, purified via SPE, then detected via LC-MS/MS analysis*



RESULTS

	Hybrid LC-MS/MS		Direct Digestion LC-MS/MS	
Peptide	**Range (µg/mL)**	**Linear Fit (r^2)**	**Range (µg/mL)**	**Linear Fit (r^2)**
SINSATHYAESVK*	0.050 - 50	0.991	1 – 100	0.997
GLEWVAEIR	0.100 - 50	0.990	1 – 100	0.996
YASESMSGIPSR	0.100 - 50	0.992	-	-
LEESGGGLVQPGGSMK	0.100 - 50	0.992	-	-
ASQFVGSSIHWYQQR	0.100 - 50	0.991	-	-
DILLTQSPAILSVSPGER	0.100 - 50	0.991	-	-

Table 2. Calibration performance of infliximab peptides monitored by Hybrid LC-MS/MS and Direct Digestion LC-MS/MS assays.

- **SINSATHYAESVK peptide measurements of patient samples did not agree with other peptides and were excluded from Hybrid LC-MS/MS analyses*
- *Only SINSATHYAESVK and GLEWVAEIR peptides resulted in linear curves using the Direct Digestion LC-MS/MS method*

Drug Level Classification of Patient Samples (N = 89)			
Drug Level Classification	**LISA TRACKER**	**Hybrid LC-MS/MS (% Agreement)**	**Direct Digestion LC-MS/MS (% Agreement)**
Subtherapeutic	3	1 (33)	0 (0)
Intermediate	14	8 (57)	11 (79)
Therapeutic	72	80 (90)	78 (92)

Table 3. Drug level classifications.

- *Cutoffs for classification were set according to LISA TRACKER as subtherapeutic (< 1.0 µg/mL), intermediate (1.0—2.0 µg/mL), and therapeutic (> 2.0 µg/mL)*
- *In clinical practice, 1.0—1.5 µg/mL is classified as borderline subtherapeutic: 7/14 LT, 4/8 IA, and 4/11 DD fall in this category*
- *Classification of patients as therapeutic was in better agreement overall (~ 90 %) than intermediate and subtherapeutic levels*
- *Hybrid LC-MS/MS and Direct Digestion LC-MS/MS results were good with ~60 % total agreement for both assays*

Figure 2. Hybrid LC-MS/MS vs. LISA TRACKER

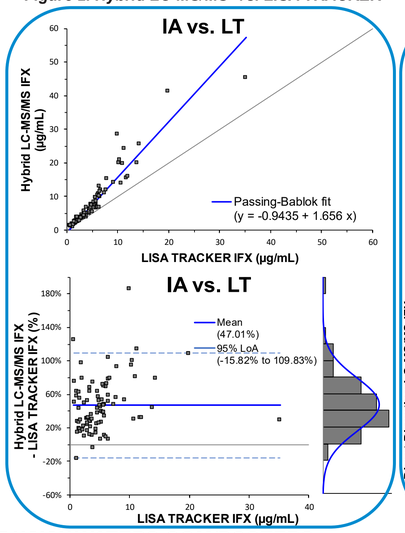


Figure 3. Direct Digestion vs. LISA TRACKER

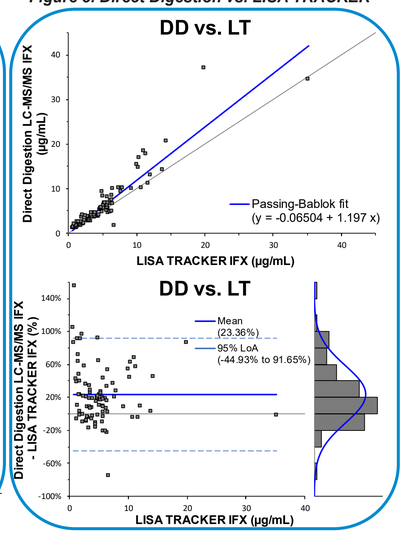
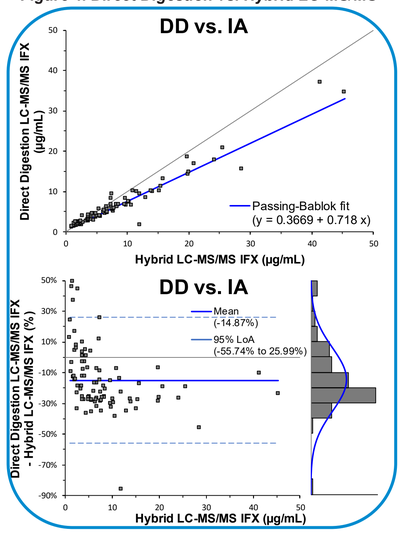


Figure 4. Direct Digestion vs. Hybrid LC-MS/MS



DISCUSSION

LC-MS/MS methods were successfully employed for the quantification of Infliximab from CD patient samples

- Calibration performance of both LC-MS/MS methods was excellent with linear fits (r^2 > 0.99) and dynamic ranges adequate for the quantification of infliximab from patient sera (Table 2)
- The SINSATHYAESVK peptide of Infliximab showed some evidence of deamidation *in vivo* (not shown here) which was identified in the hybrid LC-MS/MS assay only. This peptide was excluded from quantitative analyses for this reason
- The hybrid LC-MS/MS quantification results were proportional to the LISA TRACKER results and had a mean bias of + 47.0 % (Figure 2)
- The direct digestion LC-MS/MS assay was also proportional to the LISA TRACKER assay with a mean bias of + 23.4 % (Figure 3)
- As expected, both LC-MS/MS assays had proportional responses to each other with a mean bias of - 14.9 % (Figure 4)
- Drug levels of each patient sample were classified as subtherapeutic (< 1.0 µg/mL), intermediate (1.0—2.0 µg/mL), or therapeutic (> 2.0 µg/mL) based on each assay's quantitative results. Assay agreement is good with ~ 90 % agreement between all assays at the therapeutic level (> 2.0 µg/mL) (Table 3)

CONCLUSION

Two sample preparation approaches have been developed and employed in the quantification of infliximab from patient sera

- The addition of immunoaffinity capture improved assay specificity, enabled better agreement of peptide measurements, and improved confidence in LC-MS/MS analytical results over a direct digestion method
- The specificity inherent in LC-MS/MS assays, particularly for immunoaffinity approaches, affords better confidence in detection of analytes as compared to ELISA methods
- In the future, applying cutoffs appropriate for LC-MS/MS methodologies could generate more accurate results and better agreement in drug level classifications among quantification assays
- Multiple modifications of amino acid residues *in vitro* or *in vivo* can change the effectiveness of a biotherapeutic, and may also change the specificity of quantitative results which could effect clinical drug classifications and decisions
- Hybrid LC-MS/MS assays can be further developed to monitor the presence of anti-drug antibodies and modifications to peptides, such as deamidation

References

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