Journal of Mass Spectrometry and Advances in the Clinical Lab 32 (2024) 24–30

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Short Report

Infliximab Therapeutic monitoring by tryptic peptide LC-MS/MS method improvements lead to improved accuracy with decreased imprecision and turnaround time

Paula M. Ladwig, Ann L. Rivard, Alex Barbeln, Anthony Maus, David L. Murray, Melissa R. Snyder, Maria A.V. Willrich *

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

ARTICLE INFO

Keywords:
Infliximab
Remicade
Mass spectrometry
Tryptic peptide
Therapeutic drug monitoring

ABSTRACT

Introduction: Therapeutic drug monitoring of infliximab has become the standard of care for inflammatory bowel disease in the setting of loss of response to therapy, and occasionally in proactive therapy personalization. Measurement of infliximab by tryptic peptide HPLC-MS/MS has been available since 2015, mostly in reference laboratories.

Objectives: Here, we present method improvements to our original published method leading to a more efficient, robust, and high throughput tryptic peptide HPLC-MS/MS assay for infliximab quantitation.

Methods: Deidentified residual serum samples submitted for clinical testing were used for method comparison and infliximab was spiked into normal human serum for performance studies. Improvements included the addition of a stable isotope labeled full length infliximab internal standard (IS) replacing a surrogate IS, and immunoenrichment using Melon Gel for immunoglobulins replacing the saturated ammonium sulfate precipitation. Digestion and chromatography were optimized, and automation was added. The method improvements were validated to include precision, accuracy, reportable range, linearity, and analytical sensitivity.

Results: The digestion time was reduced from overnight to 1 h. The assay analytical measuring range (AMR) remained the same throughout improvements, 1–100 μg/mL, with linearity of $0.98x + 0.50$, $R^2 = 1.00$. Intra- and inter-assay imprecision were less than 5 % CV at four different concentrations. Accuracy was assessed with 106 patients within the AMR; Passing-Bablok Regression yielded a slope of 1.00 and a y-intercept of 0.25. Turnaround time was reduced by 1 day, and imprecision of three levels of quality control trended down after new method implementation.

Conclusions: Method improvements including automation have allowed for assay completion in half a day, improving robustness and turnaround time.

1. Introduction

Infliximab is a chimeric IgG1 kappa monoclonal antibody therapy (t-mAb) that inhibits tumor necrosis factor alpha (TNF-α), a potent pro-inflammatory cytokine with a critical role in the inflammatory responses seen in several autoimmune diseases [1]. Mechanistically, infliximab binds to soluble and membrane-bound TNF-α and initiates complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity [2]. Infliximab has been approved for use by the United States FDA for the treatment of rheumatoid arthritis, psoriatic

Abbreviations: AMR, Analytical measuring range; CV, Coefficient of variation; DTT, 1,4-dithiothreitol; HPLC, High performance liquid chromatography; HPLC-MS/MS, High performance liquid chromatography-tandem mass spectrometry; IAA, Iodoacetamine; IBD, Inflammatory bowel disease; IRB, Institutional Review Board; IS, Internal standard; LC, Liquid chromatography; LLOQ, Lower limit of quantitation; LOB, Limit of blank; LOD, Limit of Detection; MPA, Mobile phase A; MPB, Mobile phase B; MS, Mass spectrometer; NHS, Normal human serum; QC, Quality control; RPM, Rotations per minute; SIL-IS, Stable isotopically labeled internal standard; t-mAb, Therapeutic monoclonal antibody; TAT, Turnaround times; TFE, Trifluoroethanol; TDM, Therapeutic drug monitoring; TNF-α, Tumor necrosis factor-alpha.

* Corresponding author at: Division of Clinical Biochemistry and Immunology, Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

E-mail address: willrich.mariaalice@mayo.edu (M.A.V. Willrich).

https://doi.org/10.1016/j.jmsacl.2024.01.007
Received 26 October 2023; Received in revised form 15 January 2024; Accepted 31 January 2024
Available online 3 February 2024

arthritis, ankylosing spondylitis, adult plaque psoriasis, pediatric and adult ulcerative colitis, and Crohn's disease [3].

Infliximab concentration is commonly measured using immunoassays [4–6]. Infliximab quantitation by tryptic peptide high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was a novel method for the detection and quantitation of a t-mAb in clinical laboratories. When Mayo Clinic began working on the development of a tryptic peptide approach in 2012, there were few clinical studies and only a couple of laboratories offering testing by immunoassay. The infliximab test was validated and implemented in 2015 on a multiplex HPLC-MS/MS platform. In 2017, the American Gastroenterological Association's clinical decision support tool for adults with active inflammatory bowel disease (IBD) [7] provided guidance on patient management based on therapeutic drug monitoring (TDM) and spearheaded test utilization, interpretation, and volume growth. As the practice evolved and laboratories were faced with increasing testing demand, significant modifications allowed for the improvement of the overall efficiency of the test. Our laboratory has utilized HPLC-MS/MS for TDM of infliximab levels since 2015; see Fig. 1 [8].

Our aim is to outline the important advances in the field, both clinical and commercial, that have allowed for the iterative improvement of a method to remain viable in the clinical laboratory. This supports the idea that the test life cycle is not static. After development, validation, and test implementation, a long maintenance phase in clinical testing ensues. Keeping up with technology and commercial advances has made it possible to make a method less laborious, enabling higher throughput and better turnaround times (TAT). This is highly desirable and improves productivity.

2. Discussion

2.1. SIL-IS

Obtaining stable isotopically labeled-internal standard (SIL-IS) for quantitative HPLC-MS/MS measurements of proteins is often challenging. SIL-proteins are typically not commercially available or the cost to produce them is prohibitive. To overcome this challenge, when developing the original method in 2015, horse IgG was used as a surrogate internal standard (IS) for quantitation. In-house produced SIL-peptides were used as retention time standards. However, this required additional quality control measures to control for variation in the digestion process. For the original method, we monitored 2 peptides, one on the light chain and one on the heavy chain, and compared the quantitation of these peptides to ensure complete digestion before releasing patient results. A few years after implementing the infliximab method, commercially available infliximab SIL-IS became available through Promise Proteomics and Millipore-Sigma. Please refer to Fig. 1 for notable timeline events.

We assessed the benefits of utilizing infliximab SIL-IS by performing replicate measurements (N = 12) of quality control (QC) samples spanning the AMR (Table 1) using our original method with only the change of IS. This intraday precision experiment did not indicate an improvement in precision when using the infliximab SIL-IS compared to the surrogate IS. However, the accuracy of the results was much closer to the expected target value for the spiked QC samples, suggesting a slight improvement when using the infliximab SIL-IS. We hypothesized that the infliximab SIL-IS was more effective in correcting for mass spectrometer (MS) source conditions/suppression compared to the surrogate IS, as the monitored peptide from the infliximab SIL-IS has the exact same retention time as the monitored peptide from the native infliximab.

2.2. Enrichment

The availability of the infliximab SIL-IS allowed us to explore further method improvements that would not have been possible using a surrogate IS that did not always act the same as native infliximab in terms of enrichment, digestion, and chromatography. In the original method [8] we utilized selective size precipitation with saturated ammonium sulfate, which is a relatively nonspecific but widely used purification technique. This step was manual- and labor-intensive, requiring the manipulation of individual test tubes. Laboratorians had to manipulate these tubes in and out of a centrifuge, ensuring correct identification and order of the tubes, and then pour off the supernatant before reconstituting the pellet. This process took time and involved multiple vortexing steps. Another challenge was increased instrument downtime due to the need for frequent cleaning of the mass spectrometer. This was attributed to the lack of purification and the high peptide content injected into the system. Additionally, the lack of more specific enrichment required long chromatographic separation to minimize the potential for interferences. Peptides from both the light chain and heavy chain of infliximab had to be monitored, adding to the complexity of the method.

Melon Gel is a commercially available purification resin that allows for the enrichment of IgG antibodies from serum, making it a more selective purification technique compared to the previous method. The infliximab SIL-IS effectively corrects for any differences in the purification yield. Moreover, the Melon Gel enrichment process can be performed in a 96-well filter plate, which is compatible with automation. Hamilton pipetting was incorporated for positive patient identification, streamlining the process. With the use of Melon Gel purification, there was a significant reduction in overall intensity (background) and a notable decrease in the quantity of nonspecific peaks in the chromatograms. This indicates a decrease in the peptides eluting into the mass spectrometer and an overall improvement in the enrichment process, as shown in Fig. 2. With the superior and cleaner enrichment achieved through Melon Gel purification, we were able to increase our injection

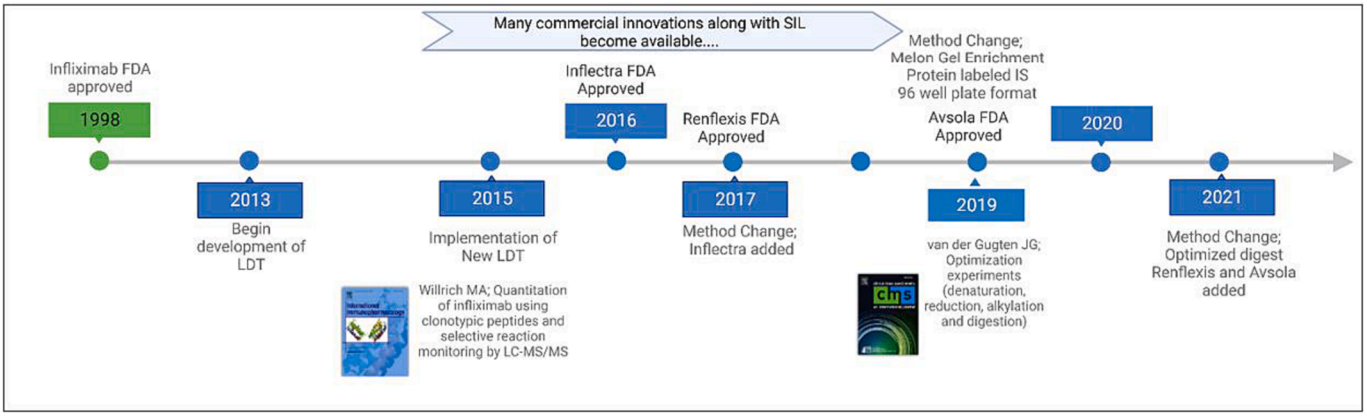

Fig. 1. Timeline outlining notable events or advances.

Table 1
Comparison of surrogate protein (horse IgG) IS to both commercial manufacturers of the infliximab SIL-IS. Results shown for the infliximab light chain y_6 transition.

	Mean Concentration (μg/mL)			% Difference from Target			% CV		
	Original IS	Millipore-Sigma	Promise Proteomics	Original IS	Millipore-Sigma	Promise Proteomics	Original IS	Millipore-Sigma	Promise Proteomics
Low QC 3 μg/mL	2.7	3.2	3.1	9	5	5	5.1	6.8	6.8
Med QC 10 μg/mL	8.5	10.1	9.9	15	1	1	2.1	3.7	3.2
Med2 QC 25 μg/mL	21.1	25.8	25.3	15	3	1	4.2	2.4	5.6
High QC 80 μg/mL	64.9	81.9	80.1	19	2	0	3.1	2.6	2.2
Mean				**14.5**	**2.8**	**1.8**	**3.6**	**3.9**	**4.5**

*Original IS = surrogate protein IS (horse IgG); N = 12 replicates.

volume from 20 μL to 50 μL. This resulted in an increased signal-to-noise ratio, further improving the sensitivity and accuracy of our measurements.

2.3. Digest

In 2019, van der Gugten and coworkers published their tryptic peptide HPLC-MS/MS method for infliximab quantitation [9]. Their method involved protein precipitation followed by a digestion process using SIL-peptides as retention time standards and internal digest controls. A significant aspect of this publication was their digestion optimization experiments, which demonstrated that the use of dithiothreitol (DTT) and iodoacetamide (IAA) was not essential for the digestion process. Additionally, they found that the digestion time could be reduced to 1 h. These findings were noteworthy as they offered possibilities for streamlining and speeding up the digestion step in the overall test method for infliximab quantitation.

DDT is used to reduce disulfide bonds, and IAA is used to cap the free thiol group on cysteines to prevent bond reformation. However, in theory, since we are monitoring peptides that are not part of disulfide bridges, trypsin digestion should free these peptides without the need for DDT and IAA. We conducted similar proof-of-concept experiments where DTT and IAA were eliminated, and the digestion time was reduced to 1 h instead of overnight. These experiments yielded comparable results. By eliminating the overnight digestion step and removing the use of DDT and IAA, we saved time for our technologists in terms of weighing these reagents daily, reconstituting them, and performing the 30-minute DTT and 1-hour IAA incubations. Our total preparation time, from thawing standards and controls to having the 96-well tray ready for the autosampler, decreased from at least 12 h to 4 h.

2.4. HPLC-MS/MS

For many years, we monitored both a primary and secondary transition for a peptide on the heavy chain and a peptide specific to the light chain [8]. The laboratory would verify the quantitation by comparing the results from both transitions and peptides. However, with the improvements made in the method, including the addition of the infliximab SIL-IS, the change to Melon Gel enrichment, and the optimization of the digestion process, the quantitation became more robust. As a result, we were able to eliminate the monitoring and quantitation of the heavy chain peptide, which eluted approximately 2 min later in the

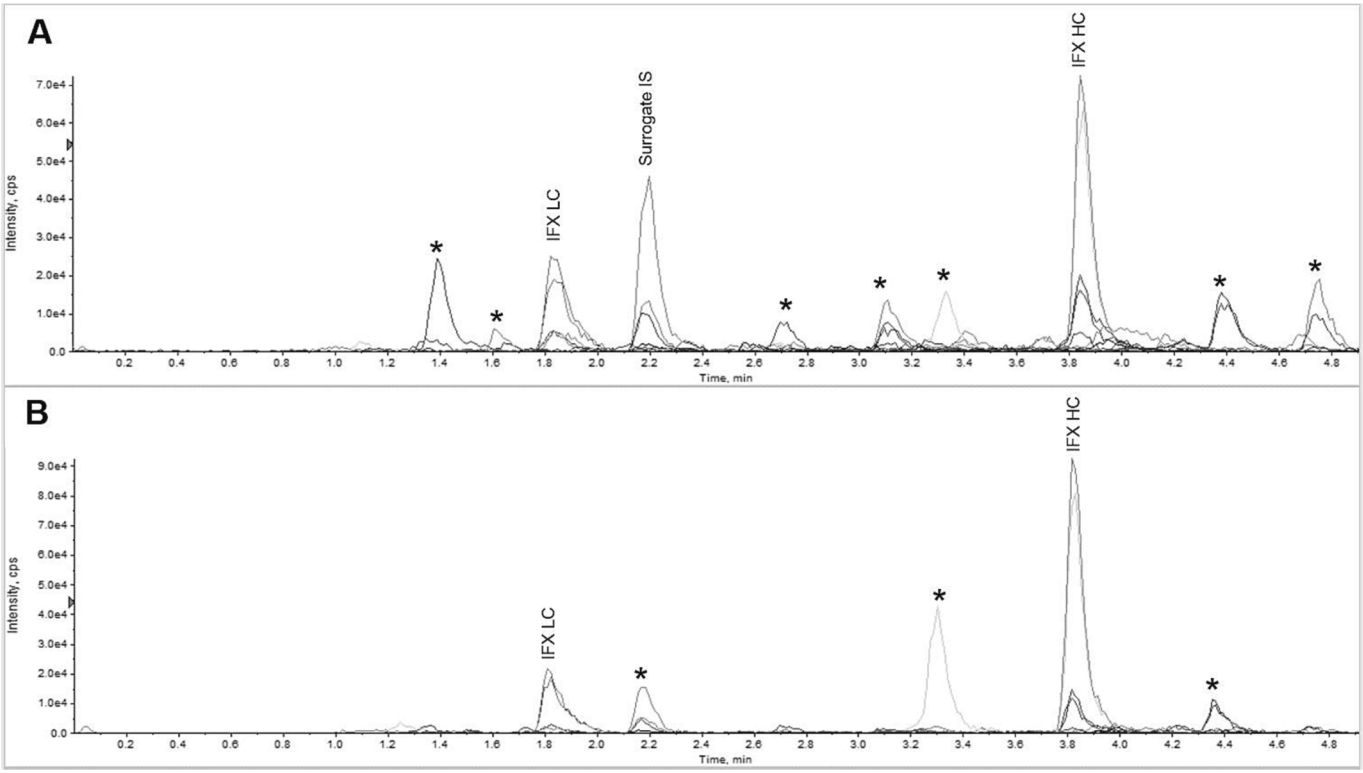

Fig. 2. Comparison of chromatograms from the low QC corresponding to the enrichment methods presented. **A)** The original method enriching with saturated ammonium sulfate (both surrogate IS and SIL-peptide retention time ISs) with a 20 μL injection and **B)** prepared with Melon Gel (incorporating the infliximab SIL-IS) with a 50 μL injection. There is not a loss in intensity for the infliximab transitions, but the quantity and intensity of nonspecific peaks (*) in the chromatograms shows that the Melon Gel purified the sample to a much greater extent.

chromatographic method. This also allowed us to eliminate four transitions (heavy chain analyte and IS) in the MS method. Instead, we now monitor only four transitions (as listed in Table 1) and increased the dwell time from 50 to 75 ms.

By no longer monitoring the heavy chain peptide, we were able to optimize a new column and significantly reduce our liquid chromatography (LC) method from 15.5 min to just 6 min.

2.5. Optimized method

Ultimately, these combined changes, as described in the following Materials and Methods section, have resulted in reduced instrument maintenance and downtime. Furthermore, there has been a reduction in the time and labor required for sample preparation. An overview of the method differences and improvements is provided in Fig. 3.

3. Materials and methods

3.1. Reagents

Ammonium bicarbonate, DTT, IAA, trifluoroethanol (TFE), trypsin and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade solvents (water, isopropanol, acetone, and acetonitrile) and Melon™ Gel was purchased from Thermo Fisher Scientific (Waltham, MA). Full-length stable isotopically labeled internal standard (infliximab $^{13}C_6$, $^{15}N_4$) was purchased from Promise Proteomics (Grenoble, France) or Sigma. Normal human serum (NHS) was purchased from EMD Millipore (Billerica, MA).

3.2. Monoclonal therapeutic antibodies

Infliximab (Remicade; Janssen Biotech) and biosimilars (Renflexis, Inflectra, and Avsola) were obtained from the institution's pharmacy. Standard curve (1–100 μg/mL) and QC (5, 10, 25 and 70 μg/mL) were prepared by spiking infliximab (Remicade) in NHS purchased from EMD Millipore.

3.3. Sample preparation

Institutional Review Board (IRB) approval was waived per our institution guidelines as no patient identifiers were used in this method validation. Deidentified clinical residual serum samples were used for method comparison. Other samples were created by spiking commercial NHS with varying concentrations of infliximab.

Sample enrichment was performed using an Agilent 96-well 2 mL 0.2-μm PES filter plate (Santa Clara, CA). In each well, 300 μL of Melon Gel pipetted into the plate followed by the addition of 25 μL of standard, QC, or unknown along with 25 μL of SIL-IS. The plate was then covered and incubated for 30 min at room temperature using a thermomixer set at 800 rotations per minute (RPM). Positive pressure was applied to move the supernatant to a 2 mL polypropylene deep well collection plate, and the filter plate was discarded. Next, 150 μL of TFE was added to the collection plate, which was then covered and incubated at 55 °C for 30 min.

After incubation, 100 μL of the mixture was transferred to a new collection plate. To this, 400 μL of 50 mM ammonium bicarbonate and 50 μL of 1 mg/mL trypsin were added. The plate was covered and

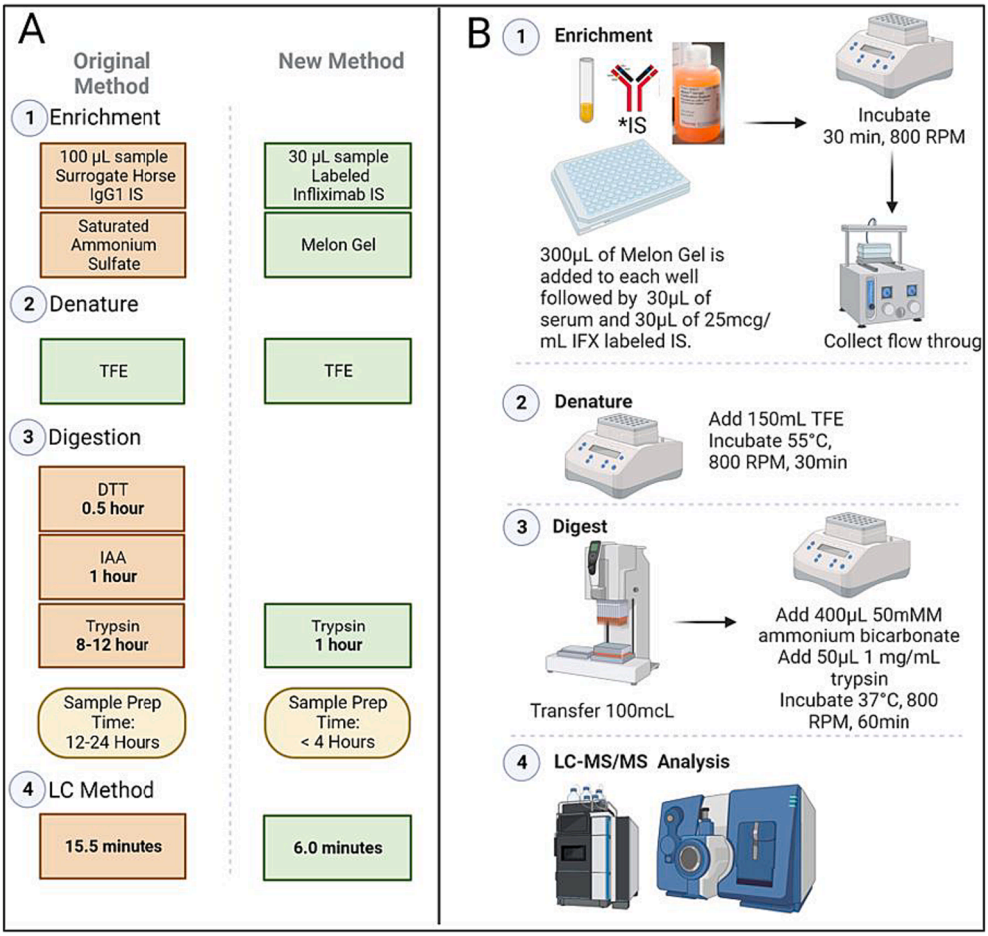

Fig. 3. A. Visual representation of the differences in the details between the original published method and the optimized method changes for infliximab quantitation by HPLC-MS/MS. B. Visual representation of the final optimized method. Figure created with Biorender.com.

incubated at 37 °C for 1 h on the thermomixer at 800 RPM. Following the digestion, 20 μL of formic acid was added to the plate to stop the enzymatic reaction. The mixture was then mixed for 1 min at room temperature on the thermomixer at 800 RPM. The plate was then centrifuged for 10 min at 3000 RPM before being placed on the autosampler.

3.4. HPLC-MS/MS analysis

A 50 μL volume of the prepared sample was injected onto a Phenomenex Kinetex 2.6-μm PS C18 (100 Å, 50x3mm) column at a flow rate of 500 μL/min. This was carried out using a Thermo TLX4 chromatography system. The HPLC method employed a six-minute runtime. It began with an initial 15-second period with 5 % mobile phase B (MPB) (0.1 % formic acid in acetonitrile) and 95 % mobile phase A (MPA) (0.1 % formic acid in clear reagent water). The method then ramped over 180 s to 20 % MPB. Subsequently, over a span of 30 s, the method further ramped to 98 % MPB, followed by a 30-second hold at that composition. Next, the method ramped down to 5 % MPB over 30 s. Finally, the method held at 5 % MPB for an additional 75 s. To achieve higher throughput, a selector valve was employed. This selector valve redirected the eluent during a 1.25-minute window starting at 2.35 min from waste to the mass spectrometer. This allowed for the utilization of 4 HPLC channels.

Peptide monitoring was performed using a Sciex API 5000 triple quadrupole mass spectrometer. The Turbo V ion source was utilized, with the following conditions: ion spray voltage:5500 V, temperature:600 °C, collision gas:12, curtain gas:40, gas 1:55, gas 2:50. Compound dependent conditions consisted of a delustering potential of 78 V and an entrance potential of 10 V. All transitions had a dwell time of 75 ms. For specific details of the MS/MS transitions, please refer to Table 2.

3.5. Method validation studies

For the optimized method, validation was conducted across two platforms: the clinical primary and backup. A total of 12 runs were performed for evaluation. Intra-assay reproducibility was assessed by setting up 20 replicates on the same run for four levels of QC (3, 10, 25 and 70 μg/mL). Inter-assay reproducibility was evaluated using the same four levels of QC along with the lower limit of quantitation (LLOQ) sample of 1 μg/mL. One replicate was tested on each of the 12 runs. Accuracy was assessed by reanalyzing 114 residual, deidentified patient samples that were selected to cover the AMR. Linearity was determined by analyzing five samples with concentrations spanning the AMR over six runs. The limit of detection (LOD) was determined by analyzing a blank sample and a sample near the LLOQ on each of the 12 runs. Carryover was evaluated by running the high standard across all four HPLC channels followed by a blank injection across all four channels on 10 runs. Bias was assessed through the spiking of three infliximab biosimilars into NHS at five concentrations across the AMR.

3.6. Data analysis

Analyst Software was used for all data processing. A 7-point linear curve with $1/x^2$ weighting (1–100 μg/mL) was generated by spiking infliximab into purchased NHS. Native peak areas were normalized to the SIL-IS peak areas. Unknown concentrations were determined by back-calculating from the established standard curve. Graphs and validation data analyses were generated with Excel. QC data and TAT reports were retrieved from clinical files from 2020 to 2022.

4. Results

4.1. Validation

Intra- and inter-assay imprecision was <5 % for 4 levels of QC and inter-assay imprecision for the LOQ of 1 μg/mL was 10 % (Table 3).

Accuracy was assessed by analyzing 114 residual patient samples on the optimized method. The results were compared using linear regression, with 106 data points falling within the AMR shown in Fig. 4. The comparison indicated good agreement with previously measured results. Eight results fell outside the AMR but remained in the same classification in both methods. One exception was a result of 96 μg/mL that was reported as >100 μg/mL in the new method. The Passing-Bablok linear regression analysis yielded a slope of 1.00 [95 % confidence interval 0.97–1.03] and an intercept of 0.25 [95 % confidence interval −0.46 to 0.68]. The median absolute difference between the two methods was 0.25 μg/mL, and the maximum absolute difference observed in concentrations greater than 50 μg/mL was around 14 μg/mL. These differences are considered clinically equivalent results for patient care.

Linearity was assessed by analyzing five concentrations across the AMR over six runs. The resulting linear regression equation was $y = 0.98x + 0.50$, with a correlation of $R^2 = 0.998$. Notably, 100 % of the points fell within ±15 % of the expected values, demonstrating excellent linearity; data not shown. The LOD was determined using the equation

Table 2
MS/MS transitions for the infliximab light chain.

	Q1 Mass (Da)	Q3 Mass (Da)	CE	CXP
SIL-IFX $^{13}C_6$ $^{15}N_4$ YASESMSGIPSR-y_{10}	647.80	1060.49	27.89	20.89
SIL-IFX $^{13}C_6$ $^{15}N_4$ YASESMSGIPSR-y_6	647.80	626.34	29.46	36.85
YASESMSGIPSR-y_{10} (quant)	642.80	1050.49	27.86	20.89
YASESMSGIPSR-y_6 (qual)	642.80	616.34	29.46	36.85

*Quantitation was performed using the y_6 transition for the original method but changed to the y_{10} for the final method. The precursor ion masses correspond to the +2 charge state of the peptide.

Table 3
Method validation precision.

	LLOQ	Low QC	Med QC	Med2 QC	High QC
N		20	20	20	20
Target (μg/mL)		3	10	25	75
Within run Mean (μg/mL)		3.5	12.3	31.3	80.5
Within run CV (%)		4.8 %	3.0 %	2.0 %	2.3 %
N	12	12	12	12	12
Target (μg/mL)	1	3	10	25	75
Between run Mean (μg/mL)	0.9	3.3	12.5	31.3	81.8
Between run CV (%)	10 %	4.7 %	2.6 %	3.2 %	2.6 %

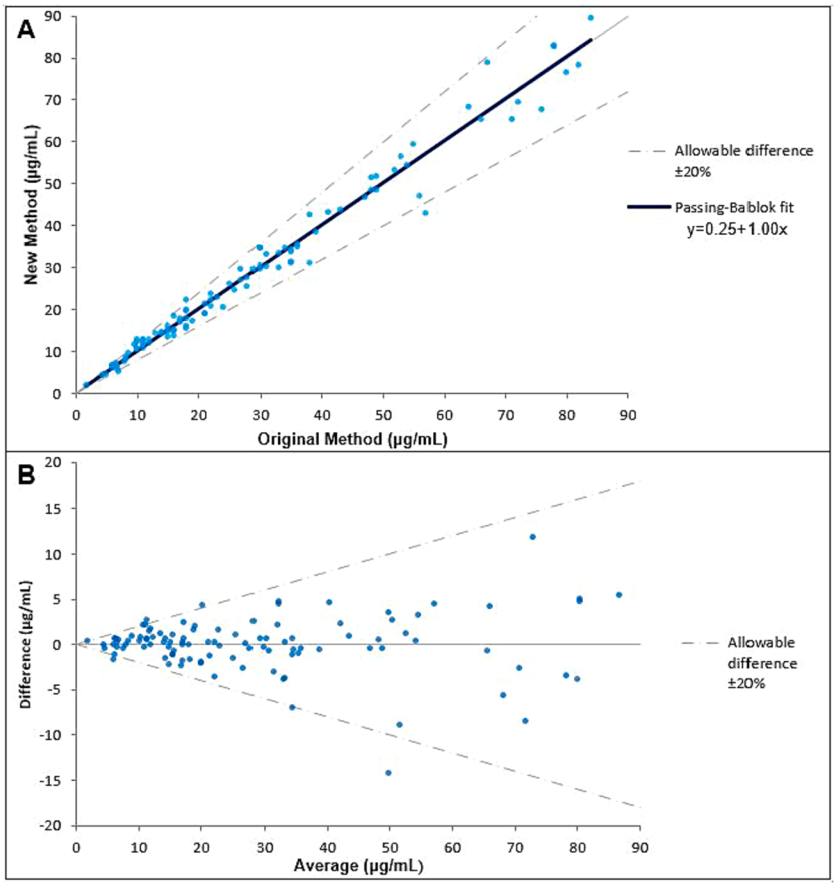

Fig. 4. A. Passing-Bablok linear regression comparison of 106 residual patients. The slope and y-intercept indicate strong agreement between the original and new method. B. Same 106 patient result comparison visualized as an absolute difference plot. Dashed lines show the allowable %difference of 20% on both plots.

LOD = LOB + 1.725 x standard deviation of the LLOQ counts. The LOD in this case was found to be 0.42 µg/mL, which is acceptable as it is less than half the LLOQ of 1 µg/mL. Carryover was assessed after the highest calibrator (100 µg/mL), and it was confirmed to be less than 10 % of the LLOQ peak area across all runs. This indicates minimal interference or contamination between the runs, ensuring the accuracy and reliability of the results.

To assess the long-term performance and bias of the new method, we obtained pharmacy stocks of a new lot of Remicade, as well as infliximab biosimilars including Renflexis, Inflectra, and Avsola. These four stocks were spiked into NHS at concentrations spanning the AMR. The measured concentrations were then compared to the expected concentrations. The resulting slopes obtained from linear regression analysis

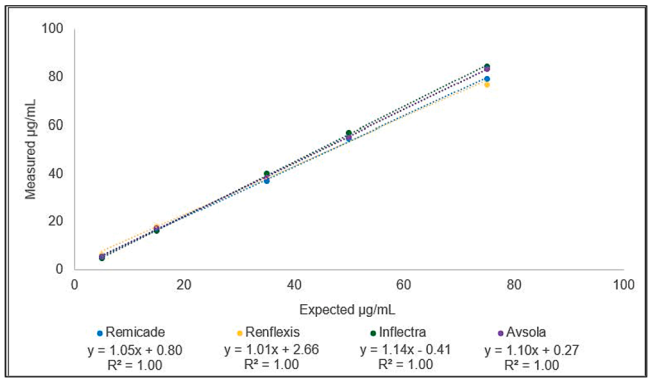

Fig. 5. Biosimilars test performance against the reference product Remicade used in a standard curve.

demonstrated less than 15 % disagreement from the target values (Fig. 5). These differences fall within our acceptance criteria (±15 %), which have been established based on the clinical applications of the testing. Therefore, we consider the test to be viable for the measurement of both the reference product and the biosimilars. It is important to note that these data were generated from a single preparation and measurement. To draw more definitive conclusions regarding the differences between the results and the associated clinical implications, additional measurements would be needed.

4.2. *Method improvement metrics*

We obtained QC and TAT metrics from 2019 to 2021 to provide objective evidence of the method improvements. Fig. 6 displays the %CV from all QC measurements made during the indicated months for the four levels that are routinely monitored. We observed a decrease in the variability of the QC measurements in the months following the implementation of the optimized method. Specifically, the imprecision of the QC measurements reduced from approximately 10 % to 5 %, as indicated by the QC monthly means. This improvement can be attributed to the combined effects of various enhancements made to the method, including the addition of the infliximab SIL-IS, the utilization of a more specific enrichment technique, and the associated improvements in the long-term performance of the instrument.

Another metric we monitored was the reduction in time and labor required to prepare and analyze samples. The optimized method resulted in a significant decrease in TAT of approximately 0.75 days on average (Fig. 7). A two-tailed *t*-test yielded a p-value of 0.0014, indicating that the change in TAT was indeed statistically significant. This improvement allows our clinical laboratory to accommodate an

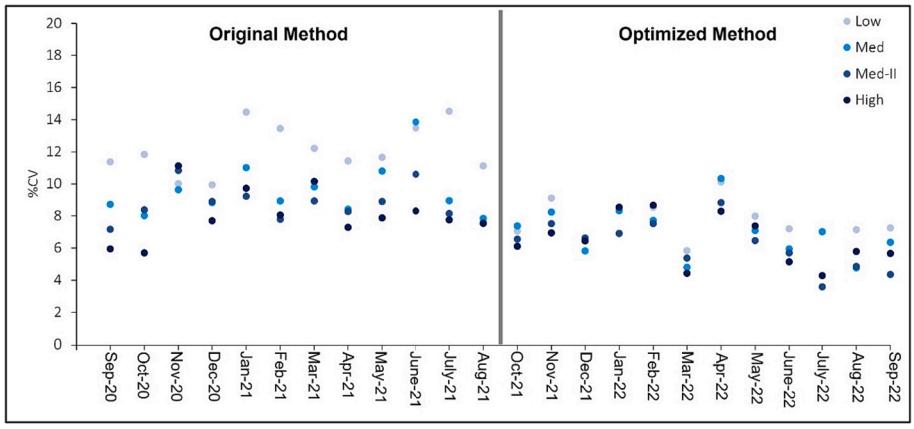

Fig. 6. Imprecision of QC across time. After implementation of the method changes presented herein the imprecision was reduced. Note: The optimized method was implemented in the middle of September 2021. Therefore, only a relatively small number of analyses were conducted on each method and this month was omitted from this chart.

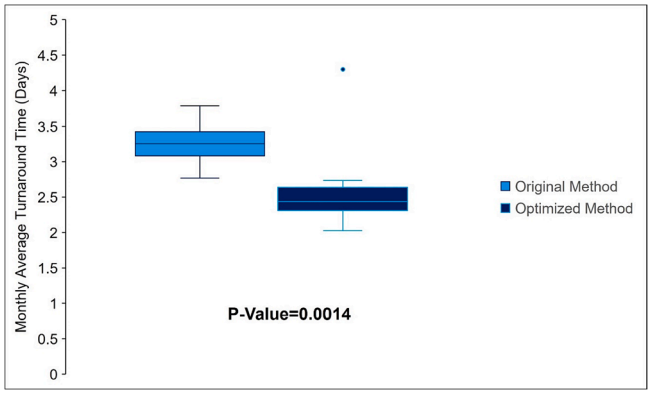

Fig. 7. Distribution of average monthly TAT for the year prior to and after implementation of the optimized method.

increased test volume, enabling clinicians to make faster decisions and ultimately enhancing patient care. It should be noted that the high outlier in TAT for the optimized method, observable in the first month after implementation, can be attributed to the training of laboratory staff and the transition of laboratory procedures to the optimized method.

5. Conclusion

The benefits of TDM for patients on infliximab is widely established. However, developing and implementing sensitive, specific, and robust methods that allow for high throughput can pose significant technical challenges. This work demonstrates the benefits of using SIL full length protein ISs and selective sample preparation techniques. These improvements optimize both the digestion process and HPLC-MS/MS parameters when performing quantitative measurements of peptides derived from infliximab. By optimizing and validating the method, we were able to improve precision and accuracy, coupled with a decrease in TAT. These advancements offer significant benefits to clinicians and patients alike.

CRediT authorship contribution statement

Alex Barbeln: Methodology, Validation, Writing-Reviewing and Editing. **Paula Ladwig:** Conceptualization, Methodology, Validation, Writing-Original Draft. **Anthony Maus:** Methodology, Writing-Original Draft. **David Murray:** Writing-Reviewing and Editing. **Ann Rivard:** Methodology, Validation, Writing-Reviewing and Editing. **Melissa Snyder:** Writing-Reviewing and Editing. **Maria Willrich:** Project administration, Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mayo Clinic has financial interest related to this research, and patents to the infliximab test have been issued. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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