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Qualification of a LC-HRMS platform method for biosimilar development using NISTmab as a model

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ABSTRACT

Biosimilars are a cost-effective alternative to biopharmaceuticals, necessitating rigorous analytical methods for consistency and compliance. Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) is a versatile tool for assessing key attributes, encompassing molecular mass, primary structure, and post-translational modifications (PTMs). Adhering to ICH Q2R1, we validated an LC-HRMS based peptide mapping method using NISTmab as a reference. The method validation parameters, covering system suitability, specificity, accuracy, precision, robustness, and carryover, were comprehensively assessed. The method effectively differentiated the NISTmab from similar counterparts as well as from artificially introduced spiked conditions. Notably, the accuracy of mass error for NISTmab specific complementarity determining region peptides was within a maximum of 2.42 parts per million (ppm) from theoretical and the highest percent relative standard deviation (%RSD) observed for precision was 0.000219 %. It demonstrates precision in sequence coverage and PTM detection, with a visual inspection of total ion chromatogram approach for variability assessment. The method maintains robustness when subjected to diverse storage conditions, encompassing variations in column temperature and mobile phase composition. Negligible carryover was noted during the carryover analysis. In summary, this method serves as a versatile platform for multiple biosimilar development by effectively characterizing and identifying monoclonal antibodies, ultimately ensuring product quality.

1. Introduction

Biopharmaceuticals are widely applied in the treatment of various diseases, and to date, over 100 mAbs have been approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [1,2]. However, their complexity and development costs contribute to their high prices [3]. Biosimilars offer a cost-effective solution as they are designed to be highly similar to innovators, providing comparable safety and efficacy at a lower cost [4,5]. The goal of reducing healthcare expenditures and improving public health can be achieved by cost-effective large-scale production of biosimilars with meticulous process controls and extensive product characterization [6,7]. It is crucial during regulatory evaluations of biosimilars to prove batch-to-batch consistency with robust evidence to demonstrate comparable quality, efficacy, and safety with the innovator [8–10]. The Quality Module of the Common Technical Document (CTD) plays a vital role during biosimilar approval, following guidelines from the ICH [11]. Characterization assays within the module establish the physicochemical and functional properties of the biosimilar, identifying critical quality attributes (CQAs) that must closely resemble the reference product [12,13]. Given the complexity of biosimilars, advanced analytical technology may be required to assess certain CQAs and meet the stringent standards set by regulatory agencies. This ensures the biosimilar serves as a safe and cost-effective alternative [14,15].

Establishing the identity and determination of the primary structure of antibody drugs is a fundamental requirement set by regulatory agencies [10,13]. Various methods are available for this purpose, including intact mass analysis and liquid chromatography mass spectrometry (LC-MS) based peptide mapping [16]. Among these methods, LC-MS based peptide mapping has emerged as a reliable and efficient approach for characterizing and ensuring the quality of therapeutic proteins. LC-MS based peptide mapping facilitates a thorough

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examination of a protein's primary structure, detecting of post-translational modifications (PTMs) and sequence variants [17]. In the later stage it can also employed in the context of the multi-attribute method (MAM) in QC [18]. In the early stages of a biosimilar development program, it is essential to begin by assessing the primary sequence identity and PTM. Biopharmaceutical manufacturers often work on multiple products simultaneously. To assess several products concurrently, a comprehensive LC-MS based peptide mapping approach proves invaluable as a research tool for gaining a deeper understanding of complex biopharmaceuticals [19]. Regulatory agencies have guidelines for validating LC-MS methods, but the instrument's complexity and various sample types make validation challenging. Ongoing efforts are being made to ensure LC-MS methods meet the rigorous standards required for use in the biopharmaceutical industry [20–23].

Nonetheless, there remains a noticeable gap in the validation of LC-MS based peptide mapping methods [24]. Therefore, our research aims to bridge this gap by conducting a comprehensive validation of the, liquid chromatography with high resolution mass spectrometry (LC-HRMS) based peptide mapping method. This endeavor will contribute to the advancement of analytical techniques available for establishing the identity, sequence coverage, post-transcriptional modification (PTMs) of therapeutic proteins, further supporting the biopharmaceutical industry in ensuring product quality and regulatory compliance.

The National Institute of Standards and Technology (NIST) has developed and released a mAb reference materials that serve as standard references to support the characterization and quality control of therapeutic monoclonal antibodies [25]. NISTmab reference materials are meticulously characterized and thoroughly validated, offering a reliable and consistent benchmark for analytical methods and instrument calibration. They are designed to mimic the complexity of real-world mAb samples, encompassing a range of posttranslational modifications, structural attributes, and heterogeneity commonly encountered in therapeutic mAbs. By standardizing measurements with NISTmab, data reliability and consistency can be achieved across different laboratories [26]. NISTmab reference materials also promote comparability and consistency of results among manufacturers and regulatory agencies, enhancing confidence in the quality and safety of mAb-based therapies [27]. Taking into consideration the advantages of availability and global acceptance we have validated the in house LC-HRMS based peptide mapping method for the identification of a mAb, using NISTmab as the model protein. In addition to identity method other attributes were evaluated for i.e. sequence coverage, post transcriptional modification (PTMs) i.e. oxidation and deamidation.

2. Materials and methods

2.1. Chemicals and materials

NISTmab was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Denosumab was obtained from Amgen (Thousand Oaks, CA, USA). Trastuzumab was obtained from Roche (Basel, Switzerland). Trypsin (Gold-Mass Spec Grade), Chymotrypsin (Sequencing grade), Glu C (Sequencing grade) and Asp N (Sequencing grade) were purchased from Promega (Madison, WI, USA). LC-MS grade waters, LC-MS grade Isopropyl alcohol (IPA), and LC-MS grade formic acid (FA) were purchased from Honeywell (Charlotte, NC, USA). LC-MS grade acetonitrile (ACN), and LC-MS grade Methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium iodide (NaI, purity ≥99.5 %), Ammonium bicarbonate, Leucine Enkephalin, and Dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iodoacetamide (IAM) was purchased from Merck (Rahway, NJ, USA). MassPREP BSA (SwissProt P02769I) digestion standard, Rapigest SF were purchased from Waters (Milford, MA, USA). L-Histidine and L-Histidine HCL were purchased from LOBA Chemie Pvt. Ltd. (Mumbai, MH, IND).

2.2. Sample preparation

Prior to LC-MS analysis, the trastuzumab and denosumab samples were prepared by dissolving in water to give a final concentration of 10 mg mL−1, while NISTmab was directly used at its supplied concentration of 10 mg mL−1. A hundred micrograms of each monoclonal antibody (mAb) sample was subjected to denaturation in 0.1 % Rapigest at 85 °C for 15 min. Following cooling to room temperature, the samples were reduced by adding 50 mM DTT and incubated at 55 °C for 45 min. Subsequently, the denatured and reduced samples underwent alkylation by adding 50 mM IAM, followed by incubation at room temperature in the dark for 45 min. The resulting samples were then digested using one of the following proteases (enzyme: protein ratio): trypsin (1:20 w/w), aspN (1:200 w/w), or gluC (1:20 w/w), in a total volume of 100 μL of 50 mM ammonium bicarbonate, at 37 °C for 4 h.

For the assessment of disulfide bridges, a sample was prepared with and without DTT, and subjected to digestion using one of the following proteases: trypsin (1:20 w/w), trypsin (1:20 w/w) combined with chymotrypsin (1:100 w/w). The digestion reaction was subsequently quenched by adding 70 μL of 0.1 % FA in water. Finally, the resulting digest was centrifuged at 10,000 g for 10 min, and the supernatant was transferred to a glass vial for LC-MS analysis.

2.3. Mass spectrometry

The peptides resulting from protease digestion of the monoclonal antibodies (mAb) were separated using online reversed-phase chromatography. This separation was performed on an Acquity UPLC CSH130C18 column (1.7 μm particle size, 2.1 × 100 mm, from Waters) within a Waters Acquity UPLC H-class Biosystem, which was coupled to a Waters Xevo G2-XS QTOF mass spectrometer.

During the chromatographic analysis, the samples underwent elution over a 91-min gradient, starting from 0 % and reaching 35 % acetonitrile, with a flow rate of 0.2 μL/min. The chromatographically resolved peptides were then subjected to further fragmentation using collision-induced dissociation (CID). Subsequently, both the parent ions (MS) and the fragment ions (MS/MS) were analyzed in positive sensitivity-MS^E mode, enabling comprehensive data acquisition.

3. Method validation

To validate our LC-HRMS method, a thorough validation process was conducted in alignment with the prevailing regulatory guidelines set forth in ICH Q2R1. Specifically, for validating an identity test, the guidelines recommend an evaluation of system suitability and specificity as essential validation attributes. In addition to confirming the identity of the analyte, this method plays a critical role in facilitating sequence comparison between an innovator product and its biosimilar counterpart. Moreover, it serves as a valuable tool for the analysis of significant post-translational modifications (PTMs) like oxidation, deamidation, and glycosylation. Given that the peptide masses within the samples represent absolute values, it became imperative to meticulously assess the accuracy and precision of the method, as detailed in Ref. [28]. Furthermore, our validation process encompassed the evaluation of other vital parameters, including assessing carryover, ascertaining method robustness, and ensuring the stability of the digestion process.

The validation of the proposed method encompasses five key attributes i.e. 1) Instrument qualification standard, 2) Specificity, 3) Accuracy and precision, 4) Robustness, and 5) Carryover, which are as follows.

3.1. Instrument qualification standard

The suitability of the system was evaluated to ensure its capability to generate accurate and reliable results [29]. As part of the instrument suitability evaluation, the Mass PREP BSA digestion standard was

monitored at the start and end of each analytical run. The chromatograms were examined for qualitative similarity, with a particular focus on the signature peptides T43, T25, T55, T22, and T71. Data analysis with a maximum difference of 10 ppm error was considered acceptable when comparing the observed masses of these signature peptides to their corresponding theoretical masses.

3.2. Specificity

The evaluation of specificity aimed to determine the method's ability to distinguish potential interferences within the mass spectra peak region where the NISTmab-specific complementarity determining region (CDR) peptides were expected to be detected. To assess specificity, mass spectra of denosumab, trastuzumab, excipient, and mobile phase blank were compared with those of the NISTmab. In addition, mixed samples and cross-contamination studies were conducted to further demonstrate the method's specificity. Furthermore, the method's specificity for disulfide bond detection, specific to the NISTmab CDR peptides, was evaluated in trastuzumab and denosumab samples. These assessments were crucial in verifying that the method can accurately and selectively identify the target NISTmab peptides, while distinguishing them from potential interferences or other samples containing similar disulfide bonds.

3.3. Accuracy and precision

Six independent NISTmab trypsin peptide mapping samples were prepared, and each sample was analyzed once. Accuracy was assessed by calculating the difference between the observed and theoretical mass values, and the mass error was expressed in parts per million (ppm). The acceptance criterion for accuracy was set at $\leq$10 ppm error for the NISTmab CDR signature peptides. Precision was determined by evaluating the repeatability on the same day by one analyst for the observed masses for the NISTmab CDR signature peptides in the sextuplicate analysis. The precision acceptance criterion was set at $\leq$0.010 % relative standard deviation (%RSD). This criterion ensures that the observed masses of the peptides remain consistent and reproducible within a narrow range, indicating a high level of accuracy and precision in the analysis.

3.4. Robustness

Various factors like composition of the mobile phases, column temperature and sample storage have the potential to impact the overall performance and reproducibility of the test. Therefore, the tolerances for each of these key parameters were assessed to ensure the robustness of the method. To evaluate the robustness, a trypsin digest sample was utilized.

Reduced NISTmab trypsin peptide maps were evaluated at a lower (0.08 %) and higher (0.12 %) formic acid concentration and compared to the concentration specified in the method (0.1 %). These peptide maps were then examined for the detection of NISTmab CDR signature peptides.

The ability to detect NISTmab CDR signature peptides at a lower (60 °C) and higher (70 °C) column temperature was evaluated by comparing to the method specified temperature (65 °C).

The time duration and storage conditions for stability of digested peptides before analysis were established. Multiple aliquots from a single glycosylated reduced NISTmab trypsin digest were stored in the auto sampler, in the specified method temperature, as well as at 4 °C in a refrigerator for 24, 48, and 72 h. Additionally, the long-term storage stability of the digested peptides was evaluated by storing them at −20 °C in a freezer for 7 days.

These robustness assessments allowed for an understanding of the method's performance under varied conditions, ensuring its reliability and reproducibility in different experimental scenarios and storage conditions.

3.5. Carryover

The ICH M10 guideline emphasizes the assessment and minimization of carryover during method validation [30]. Peptide carryover can be influenced by the properties of the peptide residues. In order to verify that carryover does not compromise the accuracy and reliability of the method, an investigation was conducted. The carryover was evaluated by injecting a mobile phase blank sample immediately after the injection of the test sample. This procedure allowed for the direct assessment of any potential contamination or carryover from previous samples. By conducting this investigation, ensured the integrity of the results and the overall robustness of the analytical method.

4. Results and discussion

In the initial phases of biosimilar development, achieving a precise amino acid sequence match with the innovator product is of paramount importance. To validate the amino acid sequence of NISTmab, a comprehensive multi-enzyme approach was employed, involving the digestion of NISTmab with trypsin, aspN, and gluC. By combining the sequence coverage obtained from each enzyme, we successfully identified every amino acid within the sequence. Individually, trypsin digestion yielded a coverage of 90 %, aspN provided 89 %, and gluC contributed 65 % coverage. However, by combining the results of all three enzymes, we achieved comprehensive coverage of 100 %, which included overlapping peptides (Electronic Supplementary Table S1). Using the same methodology, we also assessed sequence coverage for trastuzumab and denosumab, both of which exhibited 100 % coverage (data not presented).

While approximately 90 % of the sequences of all antibodies are identical, relatively small differences in variable region particularly in CDRs make each antibody unique. Generally the identity is established by sequencing the variable region of the antibody [31,32]. To identify target CDR signature peptides, NISTmab variable region were theoretically digested using UNIFI® software with trypsin, aspN and gluC, Tables 1a, 1b, & 1c respectively. Light chain peptides are represented with the prefix "1:", while heavy chain peptides are indicated by "2:" Additionally, we use the abbreviations "T" for trypsin digest, "D" for aspN, and "V" for gluC. Theoretically, the light chain and heavy chain variable region of NISTmab contain 8 and 12 peptides, respectively for trypsin digest, 5 and 10 peptides, respectively for aspN digest and 2 and 3 peptides, respectively for gluC digest. Some variable region peptides, which does not include CDR sequence and were too short or too long to achieve the high specificity. These peptides were eliminated from the analysis and include 1:T4, 2:T3, 2:T9, 1:D4, 2:D7 and 2:V3&. Variable region peptides, which include CDR sequence and were too short, were evaluated with one missed cleavage. Non CDR variable region peptide 1:T8 is a common peptide present in all three reference item tested and was reported for comparison. NISTmab variable region has two disulfide bonds i.e. one in light chain between Cys23-Cys87 and one in heavy chain between Cys22-Cys97. For identification of NISTmab specific disulfide, NISTmab digested with trypsin alone and trypsin + chymotrypsin combine in reduced and non-reduced condition and specific peptide were evaluated, Tables 2a & 2b.

4.1. Instrument qualification standard

Evaluating the performance of the LC-HRMS system for protein analysis commonly involves analyzing a standard protein digest. The sensitivity of trypsin digested bovine serum albumin (BSA) makes it a frequent choice to assess instrument performance [33]. To validate the system's ability to produce consistent and accurate outcomes, a system suitability assessment [34] was executed. In this evaluation, the Mass PREP BSA digestion standard was tracked at both the beginning and end

Table 1
Theoretical peptides of NISTmab variable region. In silico digestion of NISTmab in reducing condition was performed using the UNIFI® software for NISTmab light and heavy chain variable region and their theoretical masses are shown in a. trypsin digestion, b. aspN digestion, c. gluC digestion. Light chain peptides are represented with the prefix "1:", while heavy chain peptides are indicated by "2:", "T" for trypsin digest, "D" for aspN, and "V" for gluC.

a

Peptide identity	Peptide label	Peptide sequence
Light Chain		
Non CDR Variable region peptides	1:T1	DIQMTQSPSTLSASVGDR
CDR 1	1:T2&	VTITC**SASSR**
	1:T3	**VGYMH**WYQQKPGKAPK
Non CDR Variable region peptides	1:T4	APK
CDR 2	1:T5	LLIY**DTSK**
	1:T6	**LAS**GVPSR
CDR3	1:T7&	FSGSGSGTEFTLTISSLQPDDFATYYC**FQGSGYPFT**FGGGTK
Non CDR Variable region peptides	1:T8	VEIK
Heavy Chain		
Non CDR Variable region peptides	2:T1&	QVTLR
CDR 1	2:T2&	ESGPALVKPTQTLTLTCTFSGFSLS**TAGMSVG**WIR
Non CDR Variable region peptides	2:T3	QPPGK
CDR 2	2:T4	ALEWL**ADIWWDDK**
	2:T5	**K**
	2:T6	**HYNPSLK**
	2:T7	**D**R
Non CDR Variable region peptides	2:T8	LTISK
	2:T9	DTSK
	2:T10	NQVVLK
	2:T11&	VTNMDPADTATYYCAR
CDR 3	2:T12	**DMIFNFYFDV**WGQGTTVTVSSASTK

B

Peptide identity	Peptide label	Peptide sequence
Light Chain		
Non CDR Variable region peptides	1:D1	DIQMTQSPSTLSASVG
CDR 1	1:D2&	DRVTITC**SASSRVGYMH**WYQQKPGKAPKLLIY
CDR 2	1:D3	**DTSKLAS**GVPSRFSGSGSGTEFTLTISSLQP
Non CDR Variable region peptides	1:D4	D
CDR 3	1:D5	DFATYYC**FQGSGYPFT**FGGGTKVEIKRTVAAPSVFIFPPS
Heavy Chain		
CDR 1	2:D1&	QVTLRESGPALVKPTQTLTLTCTFSGFSLS**TAGMSVG**WIRQPPGKALEWLA
CDR 2	2:D2	**DIWW**
	2:D3	**D**
	2:D4	**DKKHYNPSLK**
	2:D5	**D**RLTISK
Non CDR Variable region peptides	2:D6	DTSKNQVVLKVTNM
	2:D7	DPA
	2:D8&	DTATYYCAR
CDR 3	2:D9	**DMIFNFYF**
	2:D10&	**DV**WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK

c.

Peptide identity	Peptide label	Peptide sequence
Light Chain		
CDR 1 & 2	1:V1&	DIQMTQSPSTLSASVGDRVTITC**SASSRVGYMH**WYQQKPGKAPKLLIY**DTSKLAS**GVPSRFSGSGSGTE
CDR 3	1:V2&	FTLTISSLQPDDFATYYC**FQGSGYPFT**FGGGTKVE
Heavy Chain		
Non CDR Variable region peptides	2:V1&	QVTLRE
CDR 1	2:V2&	SGPALVKPTQTLTLTCTFSGFSLS**TAGMSVG**WIRQPPGKALE
CDR 2 & 3	2:V3&	WLA**DIWWDDKKHYNPSLKD**RLTISKDTSKNQVVLKVTNMDTSKNQVVLKVTNMDPADTATYYCAR**DMIFNFYFDV**WGQGTTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEP KSCDKTHTCPPCPAPE

of each analysis. Importantly, all the examined signature peptides (T43, T25, T55, T22, and T71) exhibited a maximum deviation (from theoretical mass) of ±3.2 ppm when compared to their theoretical masses across all analyzed sequences (Table 3). Consequently, the system met the predefined acceptance criteria of ±10 ppm.

4.2. Specificity

According to ICH Q2R1 for identity tests, the specificity parameter should be evaluated in a method validation study. To ensure that this analytical method can differentiate the NISTmab from other mAbs, we

Table 2
Theoretical disulfide peptides of NISTmAb variable region. In silico digestion of NISTmab in reducing and non-reducing condition was performed using the UNIFI® software for NISTmab light and heavy chain variable region and disulfide peptides theoretical masses are shown in a. trypsin and trypsin + chymotrypsin digestion in reducing condition, b. trypsin and trypsin + chymotrypsin digestion in non-reducing condition.

Peptide identity	Peptide label	Peptide sequence
Light Chain by Trypsin digestion		
Cys23-Cys87	1:T2	VTITCSASSR
	1:T7	FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK
Heavy chain by Trypsin digestion		
Cys22-Cys97	2:T2	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR
	2:T11	VTNMDPADTATYYCAR
Light Chain by Trypsin + Chymotrypsin digestion		
Cys23-Cys87	1:TC3	VTITCSASSR
	1:TC22	CF
Heavy Chain by Trypsin + Chymotrypsin digestion		
Cys22-Cys97	2:TC6	TCTF
	2:TC30	CAR

b.

Peptide identity	Peptide label	Peptide sequence
Trypsin		
Light Chain C23–C87	1:T2-1:T7	VTITCSASSR=FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK
Heavy Chain C22–C97	2:T2-2:T11	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR=VTNMDPADTATYYCAR
Trypsin + Chymotrypsin		
Light Chain C23–C87	1:TC3-1:TC22	VTITCSASSR=CF
Heavy Chain C22–C97	2:TC6-2:TC30	TCTF=CAR

Table 3
System suitability. The Mass PREP BSA digestion standard was injected at beginning and end of every analysis, focusing on the evaluation of signature peptides (T43, T25, T55, T22, and T71). The error observed for each signature peptide remained within the range of ±10 ppm, thereby confirming that the system's suitability aligns with the prescribed acceptance criteria.

Peptide label	Mass Error (ppm)	
	Beginning	End
1:T22	−0.2	−2.3
1:T25&	0.5	0.6
1:T43	1.6	3.2
1:T55	−0.1	0.2
1:T71&	2.9	1.6

used the same methodology and parameters setting of NISTmab and analyzed denosumab and trastuzumab. As shown in Fig. 1a, b & 1c, 18 variable region peptides in trypsin digest, 13 variable region peptides in aspN digest and 3 variable region peptides in gluC digest can be identified in NISTmab, but these NISTmab specific peptides could not be detected in the denosumab and trastuzumab. Also, NISTmab variable region specific peptides could not be detected in mobile phase and excipient blank (RT data depicted in electronic Supplementary Tables S2, S3, S4). Conclusively, all the above demonstrated that the method is capable to differentiate NISTmab specific variable region peptides.

To ensure that this analytical method can accurately differentiate and identify the NISTmab in the presence of other components, we spiked denosumab and trastuzumab into NISTmab in equal ratio. From Fig. 1a, b, & 1c, the 13 variable region specific peptides in trypsin, 9 variable region specific peptides in aspN and 3 variable region specific peptides in gluC were detected. This can successfully identify the five CDRs out of the six CDRs of NISTmab, which indicated that identification of NISTmab was unaffected by the presence of denosumab and trastuzumab.

The inherent specificity of the CDR to individual mAbs enables the use of a single CDR as a robust means of distinguishing between different mAbs. This capability was demonstrated through visual inspection of total ion chromatogram (TIC), which, as depicted in Electronic Supplementary Figs. S1–S4. TIC visually revealed distinct peaks in the chromatograms of trastuzumab and denosumab compared to NISTmab, allowing us to use visual inspection of TIC to differentiate changes in NISTmab. Simultaneously the CDR peptides of denosumab and trastuzumab were monitored. The method demonstrated high specificity to denosumab and trastuzumab which meant that it can be used to identify cross-contamination from other mAbs produced in one site. This differentiation can be successfully accomplished using either one/combination of the digestion system that we have evaluated. Notably, for NISTmab, trypsin stands out as the preferred choice, as it can effectively identify five out of the six CDRs.

Additionally, we extended our monitoring to NISTmab specific disulfide bonds. As illustrated in Fig. 1d and e, we successfully detected the heavy chain disulfide linkage Cys22-Cys97 in both the non-reduced peptide mapping (NRPM) trypsin and trypsin + chymotrypsin digests, along with another peptide corresponding to the light chain disulfide linkage Cys23-Cys87 detected in the NRPM trypsin + chymotrypsin digest (RT data depicted in electronic Supplementary Table S5). This underscores the method's proficiency in identifying NISTmAb specific variable region disulfides, with one such disulfide situated in the light chain and another in the heavy chain.

4.3. Accuracy and precision

Although ICH Q2R1 does not address the evaluation of accuracy and precision in the validation of identity methods, we felt the necessity to assess these parameters. We believe it is crucial to consider accuracy and precision since peptide masses are absolute values that should remain constant throughout the analysis. All six TIC overlays of the preparations are reported in Electronic Supplementary Fig. S6, and they all align well with each other. To determine accuracy, we measured the variance between the observed and theoretical mass values of the peptides, calculating the mass error in ppm. Precision was evaluated by examining the consistency of observed peptide masses in the sextuplicate analysis. Our findings, depicted in Table 4a, indicate that out of the 18 NISTmab specific trypsin digest peptides identified for specificity, a total of 15 were successfully and reliably detected. However, we were unable to detect three trypsin digest unique NISTmab heavy chain peptides, namely 2:T3, 2:T4-5, and 2:T12, in two of the injections. Nevertheless, the absence of these peptides did not compromise the overall identity of the NISTmab. The 2:T3 peptide, which is a small non-CDR variable

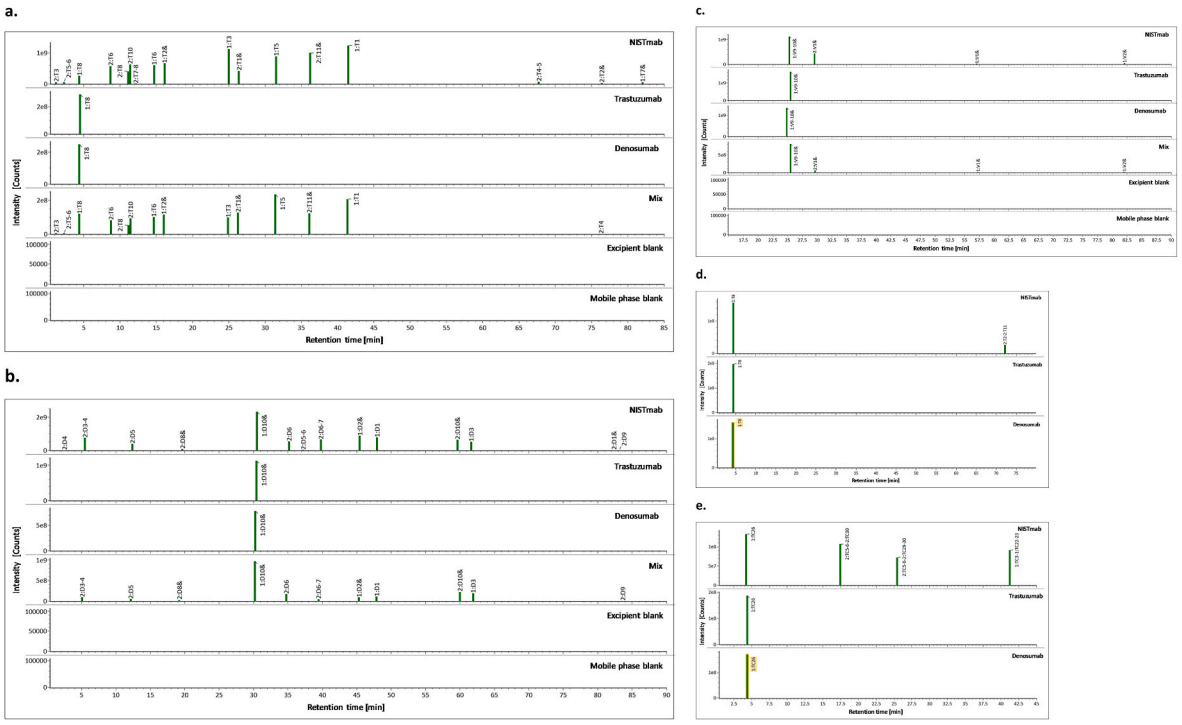

Fig. 1. Method specificity. This analytical method differentiates NISTmab specific variable region peptides. Component plot of reduced peptide map of a) trypsin digested samples, b) aspN digested samples and c) gluC digested samples shows absence of NISTmab specific variable region peptides in other mabs, mobile phase and excipient blank and presence in mix sample. Component plot of non-reduced peptide map of d) trypsin digested samples and e) trypsin + chymotrypsin combine digested samples shows absence of NISTmab variable region specific disulfide peptides in other mabs.

region peptide consisting of only five amino acids, and the 2:T4-5 peptide, which represents a single miss cleave peptide from heavy chain CDR 2, can be identified through other peptides, specifically 2:T4 and 2:T5-6. As for the 2:T12 peptide, which represents the heavy chain CDR 3, it was not detected in the mixed sample due to its lower abundance. However, peptides representing the heavy chain CDR 3 were detected in higher abundance through aspN digestion, ultimately not impacting the overall identity of the NISTmab. Additionally, the NISTmab glycosylated reduced trypsin peptides exhibited a maximum error of NMT ±2.42 ppm when compared to the theoretical masses. Furthermore, the peptides that underwent six injections demonstrated an observed mass %RSD of ≤0.010 %. The analysis consistently demonstrated a sequence coverage of 90 % across all six preparations, as illustrated in Electronic Supplementary Table S7. Chromatographic parameters, including RT and the extracted ion chromatogram (XIC) were closely monitored across six preparations, revealing %RSD values of less than 3 % for RT and below 15 % for XIC area, as outlined in Table 4a. Our investigation for PTMs reported in Table 4b, we successfully identified 7 out of 8 methionine oxidation sites, with strong agreement among the six preparations and minimal standard deviation (SD). Similarly, 20 out of 22 asparagine deamination identified, displayed consistency between preparations with low SD. We also observed repeatability with a low SD in percent glycoform at the asparagine 300. The percentage of major glycoforms was found to align with published data [25].

4.4. Robustness

In robustness measured changes to the standard protocol were performed and tested out to ensure that changes in sample preparation and sample analysis as well as the storage conditions used do not affect the analytes. Formic acid (FA) is a preferred ion pairing agent in LC-HRMS analysis for peptides and proteins due to high efficiency in ionization. However, in LC-HRMS analysis, it also effects the peptide elution profile [35,36]. FA compositions in mobile phases were altered to test if the peak profile and detection of NISTmab CDR signature peptides were affected. Water and ACN containing 0.08 % FA and 0.12 % FA, were evaluated (Fig. 2a and b with RT details available in Electronic Supplementary Table S5). It showed that compared with 0.1 % FA, peptides generally eluted earlier in 0.08 % FA, while most of the peptides were eluted later in 0.12 % FA. Similarly, variation of column temperature also impacted marginally on RT of the peptides. Comparing with 65 °C, at 60 °C peptides eluted later and at 70 °C peptides eluted earlier (Fig. 2a and RT data available in Electronic Supplementary Table S6). In terms of identifying peptides of CDR (Fig. 2b), it showed that fluctuation of acidity or column temperature has no significant influence on the results.

Storing digested peptides is essential for analyzing extensive sample sets. These peptides exhibit heightened sensitivity to storage temperature, potentially undergoing structural changes involving hydrolytic cleavages and Asn deamination, which can result in irreversible damage. Additionally, peptides have a tendency to adhere to the inner surfaces of sample containers, causing analyte concentration reduction and altering sample composition [37,38]. To guarantee the integrity of processed samples, the stability of the digested peptides was assessed across diverse storage conditions. The stability of unique peptides in several storage conditions including in the auto-sampler as per method temperature and at 4 °C for 24, 48 and 72 h s and at −20 °C for 7days in freezer were evaluated (RT data available in Electronic Supplementary Table S7). As shown in Fig. 2e that a NISTmab CDR peptides were detected at different conditions, which meant that this qualitative method is robust.

Upon comparing the TIC obtained under different storage conditions (Fig. 2c and d) with the control samples, we identified the emergence of novel peaks. This visual inspection of TIC methodology was also employed to assess major PTMs. Methionine oxidation was found to be stable and mostly unaffected in all storage conditions, except for a significant increase in M361 and M431 after 72 h in the autosampler (Electronic Supplementary Fig. S8). Asparagine deamidation remained

Table 4

Accuracy and precision. The accuracy of the 15 peptides unique to the NISTmab variable region was evaluated through the mean ppm error by comparing the identified masses with their theoretical values. Additionally, precision was assessed by calculating the percent relative standard deviation (%RSD) of observed peptide masses across six injections. All the peptides met the predefined acceptance criteria for accuracy and precision. **b.** PTMs were evaluated for methionine oxidation, aspargine deamidation and glycosylation.

a.

Peptide label	Mean observed mass (Da)	Observed mass RSD (%)	Mean Error (ppm)	Acceptance criteria	Mean RT (min)	RT RSD (%)	Mean Area	Area RSD (%)
1:T1	1892.903417	0.000090	0.82	≤0.010 % RSD	41.74	0.31	1.47E+09	13.77
1:T2&	1081.532350	0.000050	1.60	&	16.12	0.43	4.87E+08	10.63
1:T3	1621.793267	0.000039	−0.68	NMT ±10 ppm error	25.06	0.47	6.29E+08	13.37
1:T5	952.535417	0.000082	0.47		31.67	0.24	7.84E+08	10.74
1:T6	786.445500	0.000065	−1.68		14.75	0.33	4.37E+08	13.17
1:T7&	4483.009317	0.000219	1.58		81.24	0.05	6.51E+06	8.58
2:T1&	599.351533	0.000077	0.68		26.66	0.44	4.52E+08	8.97
2:T2&	3700.875317	0.000084	1.10		75.74	0.14	4.53E+06	12.67
2:T4	1660.802000	0.000106	0.87		75.54	0.07	3.05E+06	11.55
2:T5-6	986.539717	0.000094	−2.12		2.15	2.87	2.16E+07	13.68
2:T6	858.445450	0.000047	−1.60		8.54	2.53	5.07E+08	12.73
2:T7-8	832.487117	0.000092	−1.88		12.31	0.52	4.40E+06	12.52
2:T8	561.359400	0.000219	−2.17		11.15	0.62	2.92E+08	14.54
2:T10	700.433483	0.000094	−2.42		11.46	0.42	4.69E+08	13.66
2:T11&	1848.791433	0.000065	1.27		36.24	0.27	8.18E+08	11.90

b.

Site of Modification	sMean (SD)
Methionine Oxidation	
LC M4 Oxidation	0.52 % (0.06 %)
LC M32 Oxidation	0.43 % (0.08 %)
HC M34 Oxidation	0.00 % (0.00 %)
HC M87 Oxidation	0.00 % (0.00 %)
HC M255 Oxidation	1.97 % (0.21 %)
HC M361 Oxidation	0.90 % (0.13 %)
HC M431 Oxidation	0.74 % (0.52 %)
HC M101 Oxidation	ND
Asparagine Deamidation	
LC N86/N87 Deamidation	1.63 % (0.09 %)
LC N101 Deamidation	0.00 % (0.00 %)
LC N159 Deamidation	0.00 % (0.00 %)
HC N62 Deamidation	0.00 % (0.00 %)
HC N78 Deamidation	1.22 % (0.07 %)
HC N86 Deamidation	0.34 % (0.14 %)
HC N162/N204/N206/N211 Deamidation	0.00 % (0.00 %)
HC N279 Deamidation	0.00 % (0.00 %)
HC N289 Deamidation	3.04 % (0.21 %)
HC N328 Deamidation	9.99 % (0.78 %)
HC N364 Deamidation	2.08 % (0.11 %)
HC N387 Deamidation	7.62 % (2.27 %)
HC N392/N393 Deamidation	0.00 % (0.00 %)
HC N424/N437 Deamidation	7.06 % (1.63 %)
HC N104 Deamidation	ND
HC N338 Deamidation	ND
Glycosylation	
HC N300 Aglycoslated	0.47 % (0.04 %)
HC N300 G0F	44.49 % (0.12 %)
HC N300 G1F	45.61 % (0.06 %)
HC N300 G2F	9.43 % (0.06 %)

unaffected for most sites, except for HC N328 and N387, which increased in all storage conditions. The 72-h autosampler storage exhibited particularly high deamidation for almost all sites (Electronic Supplementary Fig. S9).

In conclusion the compilation of the results from different conditions, suggests that there is no significant impact due to variation in mobile phases and column temperature. However, it is essential to note that there is a notable increase in observed PTMs on 72 h in the auto sampler. Therefore, it is recommended to store digested samples in the refrigerator or freezer for long-term storage, and sample sets can be analyzed up to 48 h without significant PTM concerns.

4.5. Carry-over

ICH M10 underscores the importance of evaluating and closely monitoring carryover throughout the analysis process. Should carryover arise, appropriate mitigation or reduction measures must be taken. In our study, we assessed carryover's impact by introducing a blank sample (0.1 % FA in water) immediately after a gluC-digested NISTmab sample injection. We focused on glu-C digestion due to its generation of higher molecular weight peptides, which tend to result in increased carryover compared to other enzymes we evaluated. Our assessment revealed that, in general, other enzymes exhibited lower carryover rates when compared to glu-C (data not included). The percent carryover was determined by dividing the peak areas of the respective peptides in the post-blank sample by those in the gluC-digested NISTmab sample.

Our analysis revealed that among the 34 peptides detected in gluC-digested NISTmab, nine peptides exhibited carryover in the post blank injection, with a maximum observed carryover of NMT 2.42 %. The carryover of these nine peptides does not compromise on qualitative

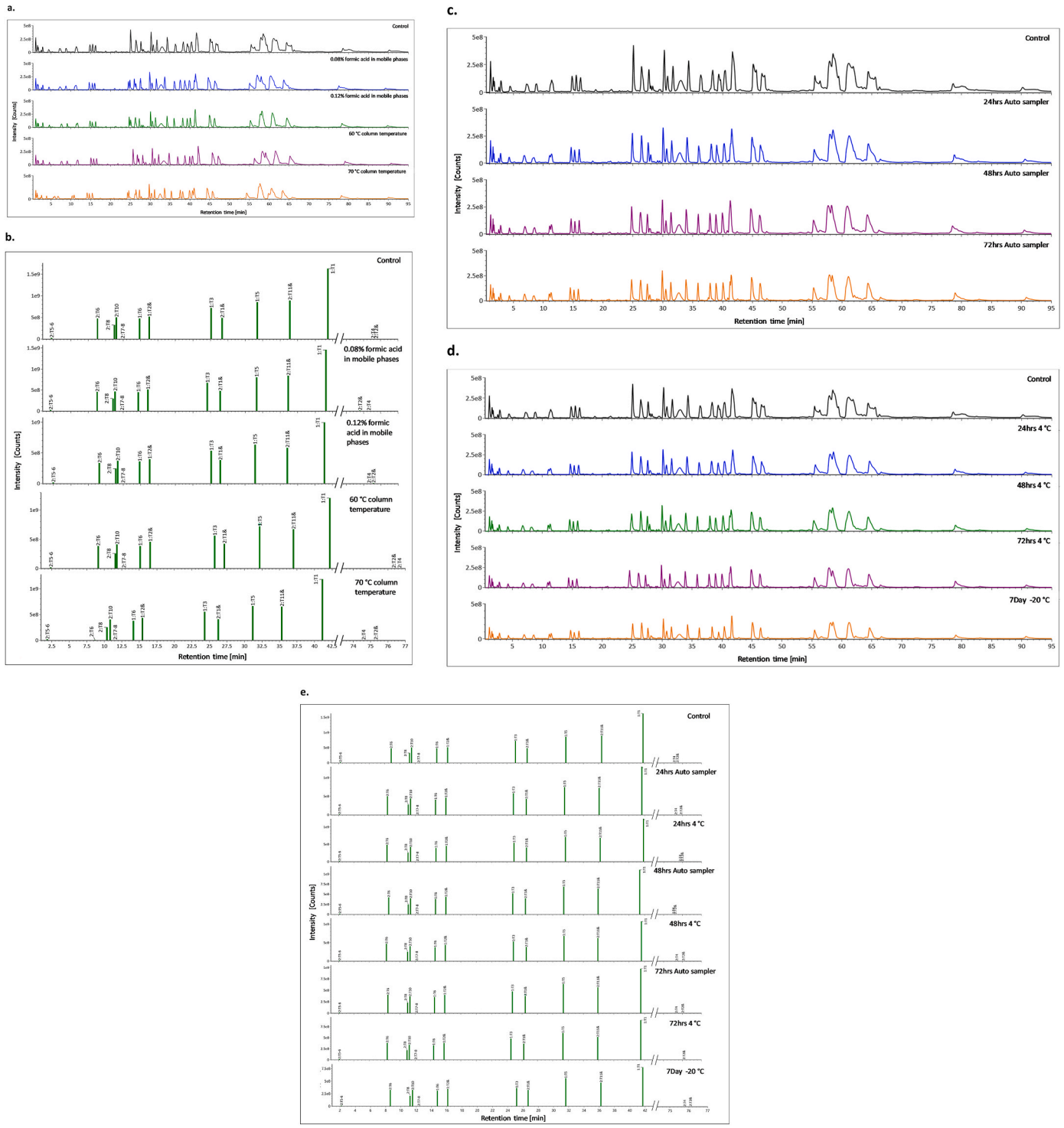

Fig. 2. Method robustness. Assessing the impact of formic acid variations in the mobile phase and column temperature, a) the total ion chromatogram (TIC) plot illustrates the peptide map profile across different formic acid concentrations and various column temperatures, b) the component plot depicts the impact on various formic acid concentrations and different column temperatures. Stability evaluation of digested samples under different conditions, c) TIC plot of samples stored in the autosampler, d) TIC plot of samples stored in a freeze and freezer, e) Component plot for NISTmab-specific variable region peptides under different storage conditions. All the tested conditions affirm the robustness of the method.

assessments of the method. However, due to variables like charge state and peptide length, substantial variations in carryover existed among different peptides, as outlined in Table 5.

Although our qualitative outcomes remained largely unaffected, we acknowledge that two peptides exhibited elevated carryover levels. Strategies for carryover reduction encompass the optimization of the digestion workflow to minimize missed cleavages, prolonging wash times for injection needles, and raising column temperatures. Transitioning from C18 to C4 reversed-phase columns have shown significant reduction in hydrophobic peptide bleeding and carry-over, resulting in more symmetrical peaks with reduced tailing [39]. However it is recommended that the potential loss of smaller hydrophilic peptides due to

Table 5
Carryover. The percent carryover was determined by dividing the peak areas of the respective peptides in the post-blank sample by those in the gluC-digested NISTmab sample.

Peptide label	Carryover (%)
1:V2&	0.56
1:V5	0.14
1:V5-6	0.22
1:V6-7	0.71
1:V7	0.70
1:V7-8&	0.32
2:V5-6&	1.47
2:V7	0.13
2:V17-18&	2.42

the less retentive column should be thoroughly assessed. We successfully addressed carryover in gluC digest by incorporating a post blank run into our analysis (data not included).

5. Conclusion

In summary, this study comprehensively validates the LC-HRMS based peptide mapping method in accordance with ICH Q2R1. The method successfully identifies CDR signature peptides and specific disulfide bonds, especially with trypsin digestion for NISTmab. It demonstrates strong specificity in distinguishing NISTmab's variable region peptides, even in the presence of other mAbs like denosumab and trastuzumab. The accuracy and precision evaluation underlined its reliability in identifying key peptides, and full sequence coverage using combine digestion system is crucial for the accurate characterization of mAbs. Additionally, it demonstrates precision in sequence coverage and PTM detection, with the visual inspection of TIC approach offering variability assessment. The study showcased the method's robustness under different conditions, with consistent results across various sample analysis and storage scenarios. Carryover assessment highlighted the importance of meticulous evaluation to ensure method accuracy. Overall, this method can serve as a platform tool for multiple mAb biosimilar development, offering a practical and cost-effective LC-HRMS solution.

CRediT authorship contribution statement

Paresh Tank: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft. **Shruti Vora:** Investigation, Writing – review & editing. **Sarita Tripathi:** Investigation. **Fatima D'Souza:** Conceptualization, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paresh Tank reports financial support and article publishing charges were provided by Zelle biotechnology pvt. Ltd. Paresh Tank and co-authors reports a relationship with Zelle biotechnology pvt ltd that includes: employment.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2024.115475.

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