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Interlaboratory Comparison of Hydrogen–Deuterium Exchange Mass Spectrometry Measurements of the Fab Fragment of NISTmAb

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ASSOCIATED CONTENT

Supporting Information

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Descriptions of materials, metrological methods, computational methods, and supplementary results. Figures of HDX-MS publications and citations versus publication year, histogram of peptide sequence lengths, sequence coverage maps, performance of instrument-software configurations, repeatability plots, $\%E_{\text{corrected}}^{\text{peptide}}(t_{\text{HDX}})$ versus $\log_{10}(t_{\text{HDX}})$ for eight peptides. Tables of instrumentation, software, peptide search methodology, and operating conditions of proteolytic, chromatographic components, and effects of peptide charge on deuterium uptake (PDF)

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Abstract

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) is an established, powerful tool for investigating protein–ligand interactions, protein folding, and protein dynamics. However, HDX-MS is still an emergent tool for quality control of biopharmaceuticals and for establishing dynamic similarity between a biosimilar and an innovator therapeutic. Because industry will conduct quality control and similarity measurements over a product lifetime and in multiple locations, an

understanding of HDX-MS reproducibility is critical. To determine the reproducibility of continuous-labeling, bottom-up HDX-MS measurements, the present interlaboratory comparison project evaluated deuterium uptake data from the Fab fragment of NISTmAb reference material (PDB: 5K8A) from 15 laboratories. Laboratories reported ~89 800 centroid measurements for 430 proteolytic peptide sequences of the Fab fragment (~78 900 centroids), giving ~100% coverage, and ~10 900 centroid measurements for 77 peptide sequences of the Fc fragment. Nearly half of peptide sequences are unique to the reporting laboratory, and only two sequences are reported by all laboratories. The majority of the laboratories (87%) exhibited centroid mass laboratory repeatability precisions of $\langle s^{\text{Lab}} \rangle \leq (0.15 \pm 0.01)\text{Da}$ ($1\sigma_{\bar{x}}$). All laboratories achieved $\langle s^{\text{Lab}} \rangle \leq 0.4\text{ Da}$. For immersions of protein at $T_{\text{HDX}} = (3.6 \text{ to } 25)^\circ\text{C}$ and for D_2O exchange times of $t_{\text{HDX}} = (30 \text{ s to } 4 \text{ h})$ the reproducibility of back-exchange corrected, deuterium uptake measurements for the 15 laboratories is $\sigma_{\text{reproducibility}}^{15 \text{ Laboratories}}(t_{\text{HDX}}) = (9.0 \pm 0.9)\%(1\sigma)$. A nine laboratory cohort that immersed samples at $T_{\text{HDX}} = 25^\circ\text{C}$ exhibited reproducibility of $\sigma_{\text{reproducibility}}^{25^\circ\text{C cohort}}(t_{\text{HDX}}) = (6.5 \pm 0.6)\%$ for back-exchange corrected, deuterium uptake measurements.

Graphical Abstract

INTRODUCTION

Over the last 28 years hydrogen–deuterium exchange mass spectrometry (HDX-MS) has developed into a powerful tool for investigating conformations, folding dynamics, and interactions among proteins including antibodies, glycoproteins, lipoproteins, membrane proteins, virus fragments, enzymes, chaperones, amyloids, fibrils, and pharmaceuticals.¹ The steady growth of HDX-MS studies is reflected in the 2247 original, heavily cited research publications appearing through 2018 (Figure S1).² In the commercial sector HDX-MS data has been used to substantiate and protect intellectual property in more than 110 United States patents since 2010.³ HDX-MS data are increasingly provided to support biologics license applications (BLAs).⁴ During 2016, 15% of BLAs to the Food and Drug Administration (FDA) included HDX-MS data.⁵ The increasing use of HDX-MS has been facilitated by rapid advances in the hardware and software that collect and analyze HDX-MS

kinetics measurements. In short, HDX-MS has emerged from the “quicksand” of frontier science,⁶ and it has become a mainstay tool in modern pharmaceutical^{4,7–26} and structural biology laboratories.^{3,23,27–35}

Major advantages of HDX-MS for characterizing the conformational dynamics of proteins are its availability to make measurements under physiological conditions, its modest sample requirements, its use of proteomic informatics that link each HDX-MS peptide sequence directly to a portion of the subject protein, and its nearly unlimited capability to characterize large proteins, for example, antibodies (~ 150 kDa)^{10,12,16,19,35} and viral capsids (~2 MDa).^{36–42} The spatial structure obtained from X-ray and NMR structure analyses, cryogenic electron microscopy (cryo-EM), and the molecular dynamics revealed by HDX data can provide an improved description of the structure–function–dynamics relationships of a protein.⁴³

HDX-MS is projected to have a role in quality control of biopharmaceuticals and for establishing dynamic similarity between a biosimilar and an innovator drug.^{7,10,22,44,45} As HDX-MS measurements are used to characterize materials that will enter commerce, customers may ask: How true and precise is the HDX-MS measurement?

Trueness is the nearness of agreement between the average of a large number of replicate measurements and a reference value.^{46,47} Although numerous approaches to the prediction of H/D exchange rates coefficient are reported,^{48–54} no means of accurately predicting exchange rates from first-principles currently exist, and no reference materials with known exchange rates are available. Thus, on the one hand, we cannot easily establish the trueness of HDX-MS measurements. On the other hand, the precision metrics of HDX-MS can be evaluated, because precision is just the closeness of agreement among measured values obtained by replicate measurements on the same or similar objects under specified conditions.⁴⁶ Precision is characterized by the components of repeatability, intermediate measurement precision (IMP), and reproducibility.⁴⁶ Determinations of reproducibility allow for variations of instruments, reagents, locations, and operators.

HDX-MS studies have reported daily repeatability ranging between 0.3% and 2.9% of maximum deuterium uptake and IMP's ranging between 1% and 9% over periods of 37 d to eight months.^{7,55–58} Cummins et al. compared HDX-MS measurements for two laboratories harmonized by employing identical procedures and equipment. For ligand-vitamin D nuclear receptor complexes immersed for 30 s in D₂O their results exhibited D-uptake repeatability and reproducibility of 0.54% for a set of 35 peptide sequences.⁵⁹

The objective of this study is to determine the reproducibility of continuous-labeling, bottom-up HDX-MS measurements (Figure 1).⁶⁰ Reproducibility is determined only through an interlaboratory comparison exercise that engages multiple laboratories in measurements of the same sample.⁴⁶ For the present unharmonized study investigators were permitted use of any HDX-MS instrumentation and software. The laboratories were not directed to report specific peptide sequences, nor were they told the deuterium uptake rates of previously observed peptides. A determination of HDX-MS reproducibility will provide a measure of its present capability for evaluations of protein commercial products.

Furthermore, an interlaboratory comparison project can itself stimulate improvements in measurement procedures and equipment.

EXPERIMENTAL METHODS

Bottom-up HDX-MS Measurements.

Laboratories conducted “bottom-up” HDX-MS experiments (Figure 1), using procedures known to provide excellent repeatability precision.^{60–63} Immersion of Fab fragment of NISTmAb reference material (PDB: 5K8A)^{64–66} sample in buffered solution (deuterium fraction, $F^{D_2O} = 0.8$ to 0.96) at temperature T_{HDX} and pD 7.48 induces D for H exchange (Figure 1A). At t_{HDX} the solution is diluted into an acidic solution ($T_{quench} \approx 0$ °C, pH ≈ 2.5 , $F^{D_2O} = 0.18$ to 0.48), containing chaotropic and reducing agents,⁶⁷ which denature the protein and reduce its disulfide bonds (Figure 1B). In this cold acidic solution, chemical exchange rates of amide sites in denatured proteins approach their minima.⁶⁸ All laboratories used the same HDX-MS kit (Figure S2) that provided buffers and reagents used during the first two steps (ref yellow/gray box of Figure 1). The kit harmonized pH, salt concentration, and reducing power (Table S1). However, specific requirements of laboratory apparatus (Tables S2–S4) required adjustments to protein concentrations and F^{D_2O} (Table S5).

Subsequently, the denatured, quenched Fab solution passes into a column containing immobilized pepsin or pepsin/protease from *Aspergillus saitoi* type XIII blend (Figure 1C).^{25,69,70} The protease digests the Fab fragment of NISTmAb ($t_{proteolysis} = (18 \text{ to } 240) \text{ s}$, $T_{proteolysis} = (0 \text{ to } 20) \text{ °C}$), and the peptides within the effluent become trapped on a guard column (Figure 1D). By flowing additional solution through the guard column for $t_{wash} = (30 \text{ to } 180) \text{ s}$, many laboratories wash out salts. Reverse-phase chromatography, conducted at $T_{LC} \approx 0$ °C, releases and separates peptides, which elute from the analytical column at $t_{LC}^{peptide}$ (Figure 1D). Electrospray ionization mass spectrometry (ESI-MS) detects the peptide ions (Figure 1E).

Alternately, immediately after D_2O incubation has completed (Figure 1B), some laboratories flash freeze the sample (Table S5). Subsequently, the sample is thawed and analyzed using a workflow similar to that depicted in Figures 2C–G.

The average mass change of each selected peptide (Figure 1F) is calculated and plotted as a function of t_{HDX} (Figure 1G). Since side-chain amides generally exchange H/D more rapidly than backbone amides,^{62,68} the deuterium labels at these positions will have equilibrated during chromatography to the natural protic isotope abundance, thus, simplifying analyses.

Separate experiments, equivalent to the HDX-MS experiment for $t_{HDX} = 0 \text{ s}$, determine the initial curated list of peptides (Figure 1F) that associates each chromatographic peak with mass spectra. To improve the veracity of peptide identifications, the operator observes MS/MS data for the eluting peptides. With reference to the known sequence of Fab of NISTmAb⁶⁵ peptide ion identification software (Tables S2 and S3) analyzes these data and

proposes an amino acid sequence, charge state (z), and confidence rank for each peptide ion fragment. The list of retention times and sequences becomes the filter through which peptide ions are selected for HDX-MS analyses. Still, the curation process continues throughout the data analysis. Curation of the list ascertains that the exchange kinetics of sequences adhere to EX2 behavior,^{1,71–73} that the D-uptake kinetics match the sequence assignments, that the LC retention times remain stable, and that peptides exhibit adequate intensity to support reliable centroid determinations and remain free of interfering ion signals.

Each laboratory conducted proteomics studies on the Fab fragment of NISTmAb and performed three HDX-MS runs. Each “run” comprises three replicant measurements, termed “reps”, at each time point, t_{HDX} . Laboratories submitted spreadsheets of centroids and information about experimental conditions to a not-for-profit data service, National Association for Proficiency Testing (NAPT), Edina, MN, which anonymized the datasheets and forwarded them to the National Institute of Standards and Technology (NIST). The data are available for study.⁷⁴

RESULTS

Laboratory Reports.

The NIST Interlaboratory Comparison Project received 15 anonymized data sets and associated documentation. The 15 data sets contain ~89 800 HDX-MS centroid measurements from 709 peptide ions, ranging from 456 to 6048 m/z and $1 \leq z \leq 8$, comprising 507 unique amino acid sequences.

A few laboratories reported sequences of the Fc fraction of NISTmAb. These sequences were expected due to trace Fc present in the Fab stock solution. Since most of the laboratory cohort did not report Fc peptides, 100 Fc peptide ions (77 Fc sequences) were deleted from the working data set. The reduced working data set for the Fab protein comprises ~78 900 centroids. The working data set contains 609 peptide ions originating from 430 sequences of the light and heavy chains.

Laboratories reported the centroid mass $\langle m(t_{\text{HDX}}) \rangle$, derived for each measurement at seven D₂O immersion times (Figure 1A), $t_{\text{HDX}} = 0, 30, 60, 300, 900, 3600$, and $14\,400$ s. By measuring peptide ions observed from the finished Fab-D₂O sample, laboratories computed the centroid mass control value, $\langle m(\infty_{\text{pseudo}}) \rangle$, corresponding to $t_{\text{HDX}} \approx \infty$ s.

Reference Peptide Maps.

Colored stripes, Figure 2, illustrate the peptide ion sequences reported by all laboratories. With reference to the legend of Figure 2, the color of each stripe indicates its coincidence frequency (ω_i^c), which is the number of laboratories reporting the i th peptide sequence (ion of any z). The x -coordinates of each stripe correspond to the start and stop indices of the peptide sequence with reference to the Fab fragment of NISTmAb. The y -coordinate position of each strip allows a unique address for each sequence. The software drawing this

map places the most frequently reported sequences (largest ω_i^c) at the lower ordinates. Where peptides of the same ω_i^c would overlap, one peptide is placed in the next higher vertical row.

Summation of all data for the Fab fragment yields a map of the heavy chain containing 221 peptide sequences (Figure 2A) and a map of the light chain containing 209 peptide sequences (Figure 2B). The sequences comprise between 4 and 60 amino acids. The set of 430 sequences reported by the laboratories has a median sequence length of 13 amino acids, and 84% of the members in the set contain between 5 and 21 amino acids (Figure S3).

Although the laboratory cohort collectively reports sequences that cover the heavy and light chains comprehensively, most laboratories reported data sets holding lower sequence coverage. The peptide sequences reported by the laboratories number between 41 and 175, corresponding to coverages from 56% to 99% for the heavy chain and from 60% to 99% for the light chain (Figure S4).

The sequence coincidence population, $M(\omega^c)$, is computed by counting the peptide sequences for each coincidence frequency, ω^c . For example, $M(1) = 245$ (represented by black stripes in Figure 2) is the population of sequences listed once across all laboratory data sets. The population $M(1)$ of the Fab fragment comprises 48% heavy-chain sequences and 53% light-chain sequences. In Figure 2 stripes of other color bars represent sequences listed twice or more across all laboratory data sets ($2 \leq \omega_i^c \leq 15$); thus, $\sum_2^{15} M(\omega^c) = 185$ shared peptide sequences. (The working data set is $\sum_1^{15} M(\omega^c) = 430$.) Across the laboratory cohort, $M(\omega^c)$ falls rapidly with increasing coincidence frequency, such that only two peptide sequences are reported by all laboratories, that is, $M(15) = 2$ (Figure S5).

The number of reported peptide sequences as a function of the six instrument-software configurations used by the laboratories was examined (Figure S6). On average, laboratories reported $\langle \bar{C} \rangle = 103 \pm 41$ (1σ) sequences. No laboratory reported peptide sequence populations falling outside the $\langle \bar{C} \rangle \pm 3\sigma$ boundaries that would identify outlying performance. This result suggests that each instrumentation-software configuration has nearly equal capacity to detect the numerous Fab fragments of NISTmAb emanating from its ESI source. Furthermore, we note that the use of a pepsin/Type XIII protease mixture by Lab 12 did not lead to identification of a superior, outlying number of peptides in comparison to the cohort using pepsin only.

Sensitivity Analyses of HDX-MS Measurements.

Knowledge the overall effect of experimental factors will guide evaluation of the measurement uncertainty. One possible error could arise if the deuterium content of peptides varies as a function of charge.^{75,76} Thus, deuterium content of centroids versus peptide charge was examined for 513 peptides ($z = +1$ to $+7$) of 106 sequences reported by 13 laboratories. No evidence of systemic, in-ESI source, intermolecular H/D exchange was observed (Table S6).

Main effects plots reveal the overall effect of several experimental factors on the HDX-MS measurement.⁷⁷ The main effects of each independent variable on the % $E_{\text{uncorrected}}$ and % $E_{\text{corrected}}$ uptake measurements are found by averaging across the levels of the other independent variables of the HDX-MS experiment. For this study main effects are computed for the factors of t_{HDX} ; *Lab*; % D_2O ; *Peptide*; T_{HDX} ; *Rep#*, repetition number; *FROZEN*, which is the flash-freezing of quenched sample prior to proteolysis; and *Run#*, run number. These computations used ~1120 measurements reported by all laboratories for the two peptides: peptide 1, ¹⁴⁹VKDYFPEPVT¹⁵⁸ of the heavy chain and peptide 2, ¹⁹⁵VTHQGLSSPVTKSFNRGEC²¹³ of the light chain.

Figure 3A displays main effects for % $E_{\text{uncorrected}}$, which are centroids processed with equation S3 and averaged from each variable. Figure 3B displays the main effects plots for % $E_{\text{corrected}}$, which was computed with equation S5. On each panel a dashed line marks the *grand mean* of deuterium exchange, which is the average of data from all laboratories for both peptides. (We note that the correction for back-exchange increases the *grand mean* deuteration of the two peptides from 32% to 59%.) Factors are ranked from left to right in accord with decreasing relative effect (RE). Below each plot a colored bar shows the $\log_{10}\text{RE}$, which indicates the sensitivity for each factor.⁷⁷ To assess the statistical significance of the variance within the eight factors, analysis of variance (ANOVA) computations were performed.⁷⁷⁻⁷⁹ In Figure 3 factors exhibiting significant relative effect ($p < 0.01$) are marked with **.

As shown in Figure 3, some factors respond in good accord with the design of the HDX-MS experiment. As examples, *mean response* (% $E_{\text{uncorrected}}$) rises from 18% to 48% between $t_{\text{HDX}} = 30$ s and $t_{\text{HDX}} = \infty_{\text{pseudo}}$ s, and peptides 1 and 2 exhibit different mean response, as expected for sequences residing in different local structural environments. Examination of the results for the procedure where samples are flash frozen (*FROZEN* = “yes”) indicate that this procedure has little effect on precision. The *mean response* % $E_{\text{uncorrected}}$ suggests a 1% improvement in deuterium recovery; however, *mean response* % $E_{\text{corrected}}$ reverses this effect by -2%. Likewise, *mean response* (% $E_{\text{uncorrected}}$) for *Run#* 1, 2, and 3 vary by less than 0.3%, which provides assurance that day-to-day system performance is nearly invariant. These same trends are also observed in plots of % $E_{\text{corrected}}$. ANOVA computations also confirm insignificant variances for factors *FROZEN* and *Run#*.

Main effects analyses reveal four nonideal responses, and ANOVA verifies the significance of the variances. % E^{peptide} should not vary with factors of *Lab* or % D_2O . Figure 3 shows that the *mean response* % $E_{\text{uncorrected}}^{\text{peptide}}$ and *mean response* % $E_{\text{corrected}}^{\text{peptide}}$ vary significantly for both factors. Moreover, the ideal HDX-MS experiment should exhibit null main effects with respect to *Rep#*; however, the *mean response* (% $E_{\text{uncorrected}}$) exhibits an ~3.5% reduction between Rep 1 and Rep 3. After back-exchange correction the mean change remains (% $E_{\text{corrected}}$) = 3.5%. Since each series of reps is executed within the same run, the diminished response is likely a symptom of incomplete removal of peptides from the chromatographic apparatus between the replicate measurements.^{80,81}

Temperature also affects acid- and base-catalyzed hydrogen–deuterium exchange rates. Amides undergo ~3× increases in exchange rates for each increment of 10 °C.⁸² In accord

with this prediction, *mean response* of $\%E_{\text{corrected}}$ (Figure 3B) increases as a function of T_{HDX} between 3.6 and 25 °C; however, some possible discordant response is displayed between 22 and 25 °C. Since the responses at 22 and 24 °C originate from two and one laboratories, respectively, it is difficult to separate effects of Lab and T_{HDX} . The main effects method does not reveal factors contributing to this incongruous response.

Determinations of Repeatability.

Repeatability measurements provide insight into the m/z measurement stability of the sample handling, mass spectrometer, software, and procedures. Figure 4 presents the fits for $\langle s^{\text{Lab}} \rangle$ versus $\log_{10}(t_{\text{HDX}})$ for $t_{\text{HDX}} = 0$ s through $t_{\text{HDX}} = \infty_{\text{pseudo}}$ s for each laboratory. The repeatability plot for each laboratory is constructed from a data set comprising between 984 and 4057 $s^{\text{Lab}}_{\text{peptide}}(t_{\text{HDX}})$ values from 41 to 175 sequences in all reported charge states (Figure S7). Uncertainty bars on the $\langle s^{\text{Lab}} \rangle$ symbols indicate the standard error of the mean, $\sigma_{\bar{x}}$. For most laboratories $\sigma_{\bar{x}} < 0.007$ Da, which corresponds to the size of the symbols in Figure 4. In summary, 87% of laboratories exhibited centroid mass laboratory repeatability precisions of $\langle s^{\text{Lab}} \rangle = 0.15$ Da, and the remaining 13% have repeatability precisions characterized by $\langle s^{\text{Lab}} \rangle = 0.14$ Da to $\langle s^{\text{Lab}} \rangle = 0.4$ Da.

Reproducibility of HDX-MS.

Reproducibility of HDX-MS is derived using sample standard deviations (equation S6) of $\%E^{\text{peptide}}_{\text{corrected}}$ for peptide sequences measured at the same T_{HDX} . The laboratories were divided into cohorts that measured $\%E^{\text{peptide}}_{\text{corrected}}$ at $T_{\text{HDX}} = (25 \pm 1)$, (21 ± 1) , and (3.6 ± 1) °C. Figure 5 shows plots of $\%E^{\text{peptide}}_{\text{corrected}}$ versus $\log_{10}(t_{\text{HDX}})$ that were reported by the nine laboratories of the (25 ± 1) °C cohort. Figure 6A plots the sample standard deviations as a function of $\log_{10}(t_{\text{HDX}})$ for peptides A–F (Figure 5) and peptides G–N (Figure S8). Each plot exhibits self-consistent D-uptake patterns. Figures 5D, S8G, S8L, and S8N exhibit D-uptake traces suggestive of kinetics that may be fit with multiple-exponential functions.⁸³ The weighted arithmetic mean of these standard deviations is $\sigma^{\text{25C cohort}}_{\text{reproducibility}} = (6.5 \pm 0.6)\%$. The nearly equal weighting of data from all laboratories ($\omega_i^c = 8, 9$) gives considerable statistical significance to this result.

The determination of $\sigma^{\text{25C cohort}}_{\text{reproducibility}}$ is also computed by expanding this analysis to include the complete subset of 130 sequences of $\omega_i^c \geq 2$ and weighting each $s^{\text{25C}}_{\text{sequence}}(t_{\text{HDX}})$ by the number of reporting laboratories. This computation yields $\sigma^{\text{25C cohort}}_{\text{reproducibility}}(t_{\text{HDX}}) = (7.4 \pm 0.3)\%$. While robust, this value reflects skew from unequal weighting given by laboratories that have reported the most sequences. Reproducibility was also evaluated for $\omega_i^c \geq 2$ sequences in other temperature cohorts (Figure 6b). Table 1 summarizes the determinations of $\sigma^{\text{cohort}}_{\text{reproducibility}}$ and the associated sample standard

deviations of all temperatures is $\sigma_{\text{reproducibility}}^{15 \text{ Laboratories}}(t_{\text{HDX}}) = (9.0 \pm 0.9) \%$, where the weighting factors are a product of ω_i^c and the number of sequences.

DISCUSSION

The NIST HDX-MS interlaboratory comparison project has found that 87% of the laboratory cohort achieved a measurement repeatability precision of $\langle s^{\text{Lab}} \rangle = 0.15 \pm 0.01 \text{ Da}$ ($1\sigma_{\bar{x}}$). All laboratories exhibited repeatability of $\langle s^{\text{Lab}} \rangle < 0.4 \text{ Da}$. The main effects analysis and measurements on 244 shared sequences determined that the reproducibility of HDX-MS is $\sigma_{\text{reproducibility}}^{15 \text{ Laboratories}} \%$. Laboratories in the $(25 \pm 1)^\circ\text{C}$ cohort realized the reproducibility $\sigma_{\text{reproducibility}}^{25\text{C cohort}} = (6.5 \pm 0.6)\%$.

This analysis used the correction for D_2O fraction and for back-exchange through measurements of $\langle m(0) \rangle^{\text{peptide}}$, $\langle m(t_{\text{HDX}}) \rangle^{\text{peptide}}$, and $\langle m(\infty_{\text{pseudo}}) \rangle^{\text{peptide}}$ on the same apparatus and with the same procedures (equation S5).^{73,84} The finishing procedure prescribed to prepare Fab- D_2O for measurements ensures that $\langle m(\infty_{\text{pseudo}}) \rangle^{\text{peptide}}$ is essentially the same for all laboratories; thus, bias in the back-exchange calculation for each coincident peptide $\omega_i^c \geq 2$ propagates equally across laboratories. Since such bias does not adversely affect the present determination of HDX-MS precision, the present results are suitable for computing the reproducibility of HDX-MS.

Intact protein mass spectrometry of Fab of NISTmAb reveals the possibility of nascent amide protons that are effectively unavailable for exchange (see Supporting Information); hence, $\langle m(\infty_{\text{pseudo}}) \rangle^{\text{peptide}} < \langle m(\infty) \rangle^{\text{peptide}}$ for a fraction of the peptides. Consequently, the $\%E_{\text{corrected}}^{\text{peptide}}(t_{\text{HDX}})$ values reported herein are not reference values for the Fab fragment of NISTmAb. The distribution of sequestered protons along the protein polymer is likely inhomogeneous.

The main effects study reveals that the peptides reported by the cohort exhibit significant deuterium loss between the first and third chromatographic gradients (reps) conducted for a run. Since the loss of deuterium exchange is observed in $\%E_{\text{corrected}}^{\text{peptide}}$ equally, the diminished response may evidence incomplete removal of peptides from the chromatographic apparatus between the replicate measurements. Adverse effects of carryover have been reported previously, and the magnitude of these effects can vary by peptide composition and column chemistry.^{80,81} Diversity of the carryover contribution across laboratories increases the uncertainty of $\%E_{\text{corrected}}^{\text{peptide}}$. Thus, minimization of carryover is essential for precise HDX-MS measurements.

The laboratory cohort reported peptide sequences with a coincidence frequency distribution containing 245 sequences of $\omega_i^c = 1$ and 185 sequences of coincidence frequencies of

$2 \leq \omega_i^c \leq 15$ (Figure S5). In view of the diverse operating conditions of the protease columns (Tables S4 and S5), the importance of this result is uncertain.

Still, proteomics studies have reported that series of pepsin digests conducted under the same conditions display a great diversity of sequences and the prevalence of unique sequences ($\omega_i^c = 1$).^{85,86} For example, Ahn et al. reported peptide identifications for a series of 31 digestions conducted at the same pH, salt concentration, temperature, and flow rate.⁸⁶ They found that a few (ubiquitous) peptides appeared in every digestion and that other peptides appeared only once in the digestion series. Unique peptic peptides outnumbered the reproducible peptides, and the number of unique peptides increased with each consecutive digestion. The number of reproducible peptides plateaued above five to six replicate digestions. In their study, a peptide was defined as “reproducible” when it appeared in 50% of digestions.

The similar populations of $\omega_i^c = 1$ and $\omega_i^c > 1$ sequences and the rapid falloff of coincidence population $M(\omega^c)$ to single-digit coincidence frequency ω^c (Figure S5) suggest that the distribution of coincident sequences may follow a modified binomial function.⁸⁷ In silico digestion by pepsin of the light and heavy chains of Fab of NISTmAb generates a powerset of nearly 8100 peptide sequences containing 4 to 30 amino acids. (This calculation used the constraints that pepsin does not cleave at the P1 position for H, K, R, and P, nor at the P2 for P.⁸⁸) When sample sizes are small compared to the total number of possible sequences, binomial functions predict that the number of unique sequences ($\omega_i^c=1$) will be greater than the number of sequences of higher coincidence frequency ($\omega_i^c > 1$), as reported in this study. For the sample sizes reported by the laboratory cohort, most binomial models predict that no peptide sequence will manifest a coincidence frequency of $\omega_i^c > 6$.

Cleavage bias of pepsin and instrument fitness may also produce subset populations of ubiquitous sequences, δ_u , that exhibit sufficient signal-to-noise ratio (S/N) for HDX-MS measurements by most laboratories. The ubiquitous sequences may account for the small set of peptide sequences with coincidence frequencies ranging $6 \leq \omega_i^c \leq 15$ (Figure S5). Thus, a modified binomial function may exist that can account for the $M(\omega^c)$ versus ω^c distribution found for the laboratory cohort.

The plethora of peptic sequences produced from the Fab fragment may cause chromatographic crowding, confounding identifications. The surfeit of species may also result in ion suppression in the electrospray source of less abundant and difficult to ionize peptides. Dependent upon operating conditions and instrument fitness, peptides exhibiting marginal S/N may sporadically fall just within and just outside acceptance criteria for centroid determinations. For proteomics measurements of the same protein, each laboratory of the cohort may acquire a distinct subset of observed peptide sequences. Sequences within each subset may appear to be selected randomly from the powerset.

Inspection of Figure 2 shows that at least 32 sequences must be measured in order to describe 95% of the Fab of NISTmAb HDX dynamics. For the quality control of a biotherapeutic product based solely on sequence-level comparisons of the deuterium content, the somewhat stochastic behavior of pepsin will add complexity, as not all sequences may be available during a measurement campaign. The quality control laboratory will usually observe the subset of ubiquitous sequences δ_u consistently, but the laboratory will likely need to repeat digestions, until the necessary peptides are measured.

CONCLUSION

The NIST HDX-MS interlaboratory comparison project has analyzed 15 HDX-MS data sets (~78 900 centroids) for the Fab fragment of NISTmAb reference material. This study finds that most laboratories achieve a measurement repeatability precision of $\langle s^{\text{Lab}} \rangle = 0.15 \pm 0.01$ Da ($1\sigma_{\bar{x}}$). Plots of repeatability can help assess HDX-MS system fitness and reveal procedural problems. Bottom-up HDX-MS generally has a reproducibility of $\sigma_{\text{reproducibility}}^{15 \text{ Laboratories}} = (9.0 \pm 0.9)\%$. The distribution of peptic peptides reported by the laboratory cohort exhibit relatively few coincidences. This low coincidence frequency distribution may require quality control methods based on bottom-up HDX-MS to perform repeated measurements to acquire a suitable number of peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bottom-up HDX-MS experiment broken into steps controlled by the NIST HDX-MS reagent kit (steps A and B in the yellow/gray box) and by each participating laboratory peptide (steps C through G). H for D back-exchange occurs during periods listed in red.

Figure 2.

Reference peptide heat map of the peptide sequences reported by 15 laboratories for Fab fragment of NISTmAb. Colors indicated on the legend denote the ω_i^c of each peptide sequence. Vertical placements of the sequence stripes are determined by sequence overlap and ω_i^c . Horizontal placements of stripes denote peptides between the N-terminal and C-terminal sequence indices for the (A) 221 sequences reported for the heavy chain and (B) 209 sequences reported for the light chain.

Figure 3. Main effects plots showing the variation of HDX-MS measurements for (A) data uncorrected for back-exchange and (B) data corrected for back-exchange. Bars denote $\log_{10}(RE)$ for each factor. Factors found by ANOVA to exert significant relative effect ($p < 0.01$) are marked with **. Dashed lines mark the mean deuterium uptake.

Figure 4. Repeatability plotted as average standard deviation $\langle s^{Lab} \rangle$ vs $\log_{10}(t_{HDX})$ for all peptide ions reported by each laboratory. When greater than the symbol, bars on each time point indicate one standard error of the mean, $\sigma_{\bar{x}}$.

Figure 5.

Plots of $\%E_{\text{corrected}}^{\text{peptide}}(t_{\text{HDX}})$ vs $\log_{10}(t_{\text{HDX}})$ for peptides measured at $T_{\text{HDX}} = (25 \pm 1) ^\circ\text{C}$. (A) LC-¹⁹⁵VTHQGLSSPVTKSFNRGEC²¹³, (B) HC-¹⁰⁶YFDVWGQGTTVT¹¹⁷, (C) HC-¹⁴⁹VKDYFPEPVT¹⁵⁸, (D) HC-¹⁶⁷TSGVHTFPAVL¹⁷⁷, (E) HC-¹⁸⁸VVTVPSSSLGTQT²⁰⁰, and (F) HC-¹⁸⁹VTVPSSSLGTQT²⁰⁰. Bars denote sample standard uncertainties larger than 1%.

Figure 6.

Determinations of HDX-MS reproducibility based on the sample standard deviation of $\%E_{\text{corrected}}^{\text{peptide}}$ for peptide sequences. (A) $s_{\text{sequence}}^{25\text{C cohort}}(t_{\text{HDX}})$ (%) as a function of $\log_{10}(t_{\text{HDX}})$ for 14 sequences measured at $T_{\text{HDX}} = (25 \pm 1)^\circ\text{C}$. Symbols denote the data from Peptides A–G (Figure 5) and Peptides H–N (Figure S8). Black squares and a trend line denote, $s_{\text{sequence}}^{25\text{C cohort}}(t_{\text{HDX}})$ (%) vs $\log_{10}(t_{\text{HDX}})$. Each black square is larger than the standard error of the mean, $\sigma_{\bar{x}} \approx 0.3\%$. (B) $\sigma_{\text{reproducibility}}^{\text{T cohort}}(t_{\text{HDX}})$ (%) vs $\log_{10}(t_{\text{HDX}})$ determined from $\%E_{\text{corrected}}^{\text{peptide}}$ for sequences measured at $T_{\text{HDX}} = (25 \pm 1)$, (21 ± 1) , and $(3.6 \pm 1)^\circ\text{C}$. Black squares and the trend line plot the weighted $\sigma_{\text{reproducibility}}^{15 \text{ Laboratories}}(t_{\text{HDX}})$ (%) vs $\log_{10}(t_{\text{HDX}})$. Each black square is larger than its standard error of the mean, $\sigma_{\bar{x}} < 0.9\%$.

Table 1.Summary of Reproducibility Determinations for Bottom-up HDX-MS for $t_{\text{HDX}} = (30 \text{ to } 14\,400) \text{ s}$

$T_{\text{HDX}}, ^\circ\text{C}$	lab cohort size	number of sequences	ω_i^c	STD ^a , %	$\sigma_{\bar{x}}^{\text{wtd}}$, %
25 ± 1	9	14	8 to 9	6.5	0.6
25 ± 1	9	130	2 to 9	7.4	0.3
21 ± 1	4	88	2 to 4	9.7	0.7
3.6 ± 1	2	26	2	22.3	3.1
wtd mean	15	244		9.0	0.9

^a cohort
 $\sigma_{\text{reproducibility}}$