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Article

Performance Assessment of a 125 Human Plasma Peptide Mixture Stored at Room Temperature for Multiple Reaction Monitoring–Mass Spectrometry

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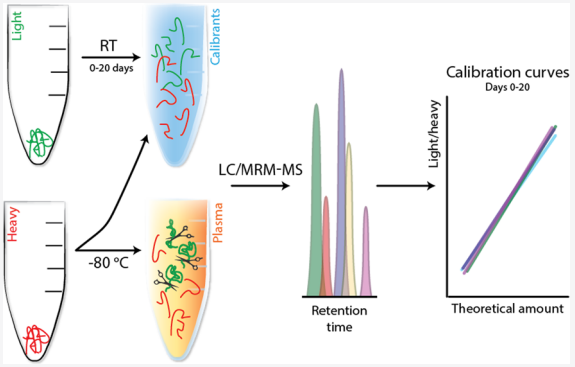
ABSTRACT: Synthetic peptides are a critical requirement for the development and application of targeted mass spectrometry (MS)-based assays for the quantitation of proteins from biological matrices. Transporting synthetic peptides on dry ice from one laboratory to another is costly and often difficult because of country-specific import and export regulations. Therefore, in this study, we assessed the impact of leaving a lyophilized mixture consisting of 125 peptides at room temperature for up to 20 days, and we assessed the effect on the quantitative performance of multiple reaction monitoring-MS (MRM-MS) assays. The findings suggest that there are no significant differences in the MRM-MS results for the time points assessed in this study (up to 20 days). All the calibration curves and quality control (QC) samples met the acceptance criteria for precision and accuracy (raw data are available via the public MS data repository PanoramaWeb, identifier: /MRM Proteomics/2020_BAK125_RT). The number of endogenous proteins quantifiable across five plasma samples was consistently between 87 and 99 out of 125 for all time points. Moreover, the coefficients of variation (CVs) calculated for the majority of peptide concentrations across all samples and time points were <5%. In addition, a lyophilized peptide mixture was transported from Canada to Iceland without dry ice. The results showed that there was no significant difference in the quantitative performance, with the determined concentrations of most proteins in the samples falling within 30% between the analyses performed on the same three plasma samples in Iceland and those in Canada. Overall, a comparison of the results obtained in Canada and in Iceland indicated that the peptides were stable under the conditions tested and also indicated that shipping lyophilized peptide mixtures without dry ice, but in the presence of sufficient desiccant material, could be a feasible option in cases where transport difficulties may arise or dry-ice sublimation may occur.



KEYWORDS: *multiple reaction monitoring (MRM), mass spectrometry, peptide, stability, proteomics, targeted proteomics, internal standards*

INTRODUCTION

The transport of biological samples worldwide has become a necessity in the modern scientific community. Collaborations involving scientists from different nations are commonplace, but they are often hindered by differences in import and export regulations between countries.[1] Maintaining the molecular integrity of biological samples in interlaboratory exchanges is typically achieved by maintaining samples at a temperature of approximately −80 °C during shipment using dry ice.[2] This is not a straightforward option in many cases, for a variety of reasons. Dry ice itself, for example, is sometimes considered to be a "dangerous good", and in several countries or regions, such as parts of Australia, there are strict regulations in place for transporting it into the country with specific couriers.[3] These restrictions often lead to the requirement of specialized carriers or routings to deliver a package containing dry ice to its final destination and can thus result in prohibitively high costs. Additionally, the large distances between countries can often lead to several days in transit, requiring either a dry-ice refill en route or much larger package sizes, again adding to transportation costs. Furthermore, in our experience, packages often undergo delays during customs clearance, which becomes a problem for both the recipient and shipper, and

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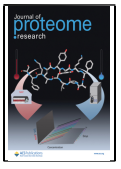

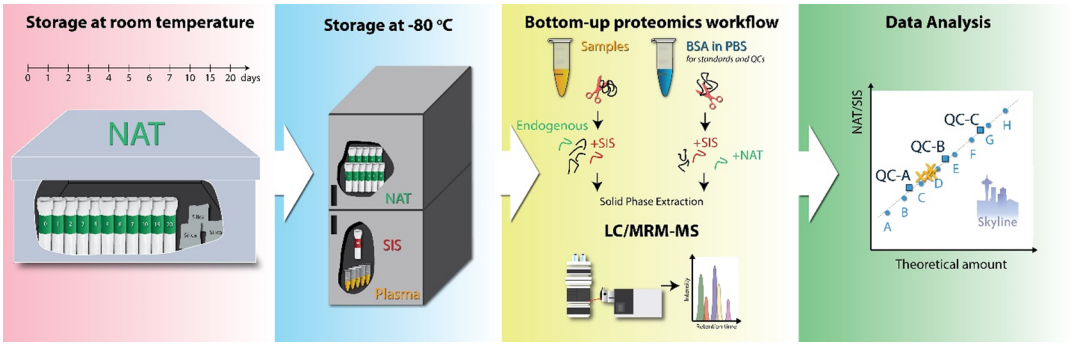

Figure 1. Stability assessment of a lyophilized peptide mixture consisting of 125 unlabeled human peptides (light, NAT) when stored at room temperature for up to 20 days. Aliquots of the peptide mixture were stored for varying time periods (0−20 days) at room temperature while controlled for humidity with silica sachets, followed by storage at −80 °C. The synthetic NAT peptide mixtures were spiked into BSA digests (the surrogate matrix) to create calibration curves and QC samples. Calibration curves were used to quantify endogenous protein concentrations in five lots of commercially available human plasma using a bottom-up proteomics workflow and LC/MRM-MS analysis. A mixture of lyophilized SIS peptides analogous to the NAT peptide mixture was stored at −80 °C until sample preparation was initiated.

can raise questions about sample integrity if the dry ice undergoes complete sublimation prior to package arrival.

Because of these challenges, it would be advantageous to transport biological samples and reagents (such as synthetic peptides) at room temperature, but proteins and peptides that are not stored at −80 °C have been shown to undergo various chemical and photochemical changes, resulting in degradation, which is further accelerated under humid conditions.[4] This has been demonstrated for single amino acids quantified from dried blood spots as well.[5] Common modifications of peptides and proteins include oxidation of cysteine and methionine residues,[6] deamidation of asparagine,[7] and hydrolysis.[8] A potential solution for slowing down these reactions and avoiding dry-ice-related shipping obstacles is to dehydrate the entire protein digest or the synthetic peptides via lyophilization, followed by shipment at room temperature. A recent study assessing the impact of shipping SW480 colon-cancer-cell protein digests in the lyophilized form without cooling found no significant impact on peptide integrity.[9]

The probability of unforeseen circumstances or logistical difficulties that could affect sample transport, along with promising literature findings regarding the integrity of lyophilized samples, has prompted this current study, where we assess the stability of peptides when shipping at ambient temperatures.

In the experiments described here, a lyophilized 125-peptide mixture designed for the quantitation of human plasma proteins via multiple reaction monitoring−mass spectrometry (MRM-MS) assays was used to determine the effect of storage at room temperature on protein quantitation. An overview of this approach is shown in Figure 1. In addition, to further assess the performance and robustness of the assays as tools for absolute quantitation in a "real-life" scenario, a set of 125 stable isotope-labeled standard (SIS) and 125 matching light (natural abundance, NAT) lyophilized peptide mixtures were shipped without dry ice from eastern Canada to Iceland. Three additional human plasma samples were shipped on dry ice from Iceland to Canada and prepared in parallel in Montreal, Canada, as well as in Reykjavik, Iceland. Samples prepared in Montreal, Canada, were run on an ultraperformance liquid chromatography (UPLC) Agilent 1290 Infinity II system interfaced to an Agilent 6495B mass spectrometer, while those prepared in Reykjavik, Iceland, were analyzed on a Waters Acquity UPLC I-Class coupled to a Xevo TQ-XS tandem-quadrupole mass spectrometer system. The results obtained in both laboratories were then compared and respective performances were evaluated.

EXPERIMENTAL SECTION

Materials

Reagents and Labware. Phosphatase buffered saline (PBS) tablets, Trizma preset crystals (pH 8.0), urea, dithiothreitol (DTT), iodoacetamide (IAA), and silica gel desiccant sachets (each containing 3 g of material) were purchased from Sigma-Aldrich. The decision to use 12 g of desiccant material was based on a previous study where the stabilities of 21 amino acids were investigated.[5] In this earlier study, two sachets containing 6 g of silica gel desiccant material each, for a total of 12 g, were used. Deep-well plates (1.1 mL) were from AXYGEN and 96-well plates (1.0 mL) were from Waters. Protein LoBind tubes and LoBind 96-well polymerase chain reaction (PCR) plates, free of DNA, DNase, RNase, and other PCR inhibitors, plates were purchased from Eppendorf. Oasis HLB μElution plates (2 mg sorbent/well, 30 μm particle size) were obtained from Waters. Ultrapure water was generated with a Milli-Q Direct 8 water purification system. Formic acid (FA), methanol (MeOH), and acetonitrile (ACN), all liquid chromatography−MS (LC−MS) grade, were purchased from Fisher Scientific or Sigma-Aldrich. Eppendorf Protein LoBind tubes were used for preparing the diluted NAT and SIS peptide mixtures.

Peptide Mixtures. The peptide standard mixtures used for this project were from MRM Proteomics Inc.'s PeptiQuant 125-protein human plasma MRM assay kits. These kits contain NAT and SIS peptide mixtures, as well as trypsin and bovine serum albumin (BSA). The synthetic proteotypic peptides contained in the two mixtures (sequences shown in the Supporting Information Table S1) serve as molecular surrogates for 125 human plasma proteins and cover several orders of magnitude in protein concentration. The peptides were selected as suitable surrogates for human proteins, as described previously,[10] and were assessed for purity and accurate concentration externally by capillary zone electrophoresis (CZE) and amino-acid analysis (AAA).[11] The detectability of each peptide spiked into human plasma had been previously established, the ionization conditions had been previously optimized empirically, and interference screening

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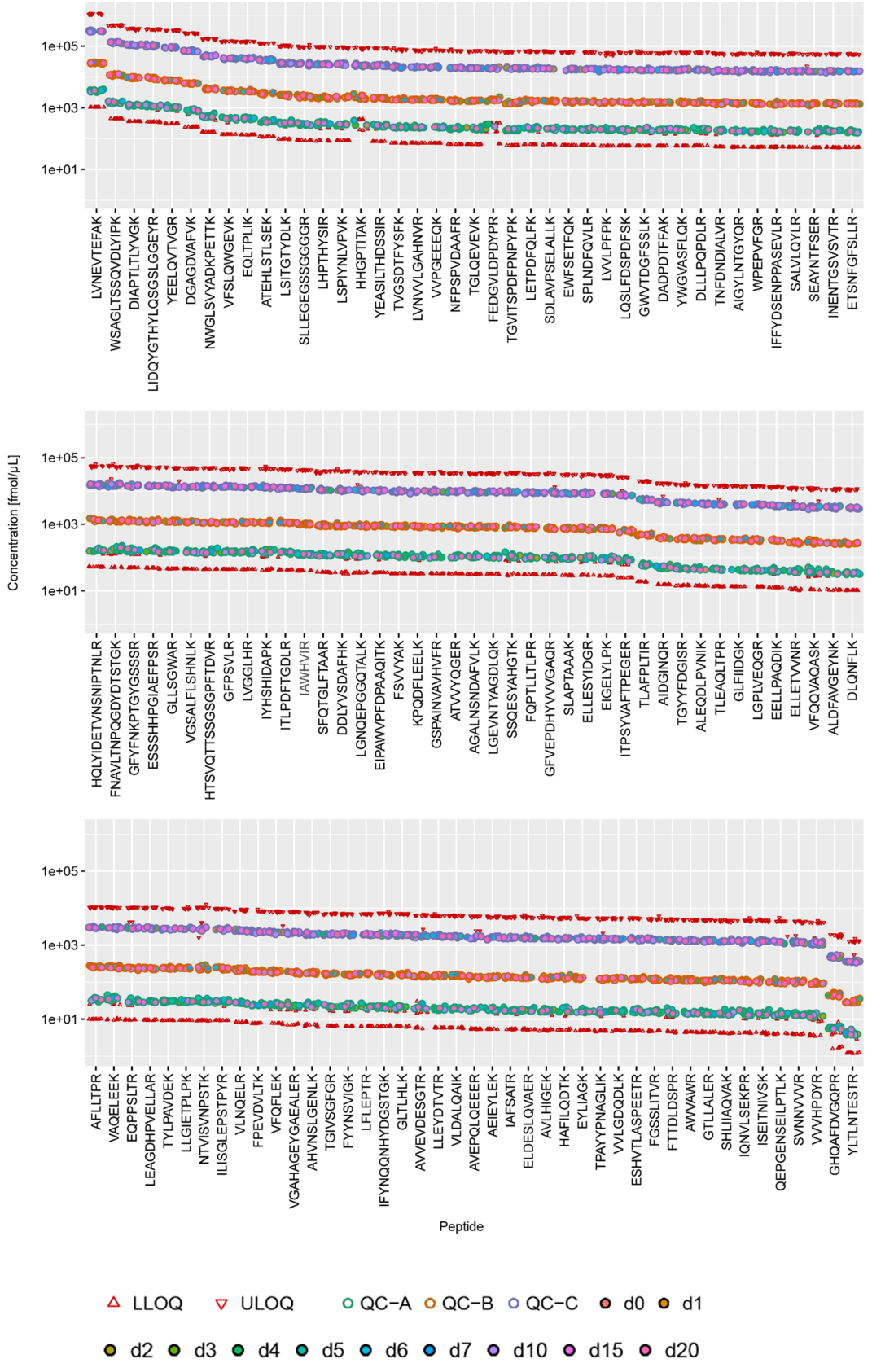

Figure 2. Overview of all peptide concentration values determined for the LLOQ, ULOQ, and QC-A, QC-B, and QC-C samples for days 0 to 20.

experiments had been performed. For each peptide, the limit of detection (LOD), the linear range, including the lower limit of quantitation (LLOQ), the upper limit of quantitation (ULOQ), and the precision, had been established in accordance with the Clinical Proteomic Tumor Analysis Consortium (CPTAC) guidelines for assay development,[12]

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J. Proteome Res. 2021, 20, 4292−4302

which are available on the CPTAC Assay portal website.[12] The peptide concentrations in the light peptide mixture were balanced[11] to the LLOQ established for each peptide.

Human Plasma. Commercially available human plasma samples from five different lots, originally obtained from whole blood donations, were purchased from BioIVT (formerly known as Bioreclamation IVT). As shown in Supporting Information Table S2, the samples were obtained from individual female donors of either Black or Hispanic ethnicity. Documentation provided by the supplier exhibited no indication of the health or disease status of the subjects, other than being in accordance with the Food and Drug Administration (FDA) regulations by testing negative for HIV 1/2 AB (human immunodeficiency virus-1 and virus-2 antibodies) and HCV AB (hepatitis C virus antibody), and being nonreactive to HBSAG (hepatitis B virus antigen), HIV-1 RNA (human immunodeficiency virus-1 ribonucleic acid), HCV RNA (hepatitis C virus ribonucleic acid), HBV DNA (hepatitis B virus deoxyribonucleic acid), and STS (serologic test for syphilis). In addition, human plasma samples from three healthy female donors were sourced and provided by our collaborators in Iceland.

Methods

Time Course Incubation at Room Temperature. A set of 10 lyophilized 125-protein human plasma NAT peptide mixtures was placed in an insulated styrofoam box at room temperature. Each aliquot was placed individually in a sealed Ziploc bag containing 10 desiccant bags with 3 g of silica gel each. An aliquot was returned to −80 °C after 1, 2, 3, 4, 5, 6, 7, 10, 15, and 20 days of incubation at room temperature. We selected 20 days as the maximum time period for this study as packages are unlikely to be in transit for longer durations. An additional never-thawed aliquot was used as "day 0." All calibration curve standards and quality control (QC) samples were hydrated and prepared as a single batch. The lyophilized SIS peptide mixtures were maintained at −80 °C throughout the experiments and were spiked into the test mixtures as normalizers to compensate for variations in sample preparation as well as changes in the ionization efficiency.

Shipment of Light and Heavy Peptide Mixtures to Iceland. Light- and heavy (NAT and SIS)-lyophilized peptide mixtures for the quantitation of 125 human plasma proteins were shipped from Canada to Iceland without dry ice. Approximately 12 g (four bags containing 3 g each) of desiccants were included, and peptide-containing tubes were tightly sealed with parafilm prior to shipping. The lyophilized peptide mixtures remained in transit for 5 days. Upon arrival, the lyophilized mixtures were stored at −80 °C until ready for analysis.

Digestion of Human Plasma and the BSA Surrogate Matrix. A total of eight individual-donor plasma samples were used. Five lots were used for room-temperature stability assessment of the 125-peptide mixture in Canada, and three different lots were also analyzed at the University of Iceland to evaluate the performance of the 125-peptide mixture after shipment without dry ice, as described above. All plasma samples, in addition to the BSA surrogate matrices, were proteolytically cleaved using trypsin. Denaturation and reduction of 10 μL of BSA (10 mg/mL) or raw human plasma were performed at pH 8 by the addition of a urea/DTT/TrisHCl buffer, resulting in final concentrations of 6 M urea, 13 mM DTT, and 200 mM TrisHCl. The samples were then incubated at 37 °C for 30 min. Next, proteins were alkylated by adding IAA at a final concentration of 40 mM and then incubated at room temperature in the dark for 30 min. Urea was diluted to <1 M by adding 100 mM Tris buffer prior to the addition of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) at a 20:1 (protein:enzyme, w/w) ratio, and the samples were incubated for 18 h at 37 °C. The digestion reaction was quenched by acidifying samples with FA to a final concentration of 1.0% FA (pH ≤ 2). The estimated concentration of the peptide mixture at this point was 1 μg/μL. The samples were kept on ice until subsequent solid-phase extraction (SPE) cleanup.

Calibration Curve and QC Sample Preparation. A surrogate matrix made of 10 mg/mL BSA in PBS was digested as described above, and then used to prepare calibration curve standards and QC samples for the 11 time points. The lyophilized NAT peptide mixtures from the 11 kits were each reconstituted in 60 μL of 30% ACN/0.1% FA to a final concentration of 400x LLOQ/μL. Each NAT peptide mixture stock was serially diluted with 30% ACN/0.1% FA to yield eight concentrations, 100, 40, 16, 4, 2, 0.5, 0.25, and 0.1× LLOQ/μL, and generate standard curves. The concentration range details for the standard curves generated for each peptide are provided in Supporting Information Table S3. The QC samples for each curve at each time point were prepared by diluting the 400x LLOQ/μL NAT peptide stocks to the final concentrations of 0.4, 3, and 30× LLOQ/μL, for QC-A, QC-B, and QC-C, respectively. Three replicates of each QC sample concentration were prepared for each curve and were analyzed along with the five plasma digest samples.

SIS Addition and Solid-Phase Extraction. Each of the 11 SIS peptide mixture aliquots, corresponding to the kits used, were rehydrated in 450 μL of 30% ACN/0.1% FA and were then pooled to be used as normalizers for all plasma samples, calibration curve standards, and QC samples.

Eight-point calibration curves were prepared in 1.1 mL 96-well AXYGEN plates by combining 10 μL of SIS peptide mixture, the level-specific amount of light peptide mixture corresponding to each calibration curve standard, and 75 μL (or 40 μL for samples processed in Iceland) of the digested surrogate matrix (BSA). Similarly, 75 μL (or 40 μL for samples processed in Iceland) of surrogate matrix, 10 μL of SIS peptide mixture, and the corresponding level-specific amount of light peptide mixture were used to prepare QC samples A through C in triplicate for each of the 11 calibration curves. A 40 μL aliquot of each human plasma digest sample was spiked with 10 μL of the SIS peptide mixture along with 10 μL of 30% ACN/0.1% FA. All QC samples, calibration curve standards, and plasma samples were brought to 600 μL with aqueous 0.1% FA, before each sample was cleaned by SPE using a Waters Oasis HLB μElution plate.

SPE sample processing was performed, as described previously.[10] Briefly, wells were conditioned with MeOH and washed with aqueous 0.1% FA. QC samples, calibration curve standards, and plasma samples were loaded onto the HLB μElution plate and washed with LC−MS-grade water. Elution was performed by adding a highly organic solvent, 50−70% ACN/0.1% FA, to all wells. The concentrated eluate was then evaporated using a speed vacuum concentrator and stored at −80 °C until MS analysis. Plasma samples, calibration curve standards, and QC samples were then resolubilized and analyzed on an Agilent 1290 Infinity II coupled to a 6495B triple quadrupole (QqQ) MS system in Montreal or on an

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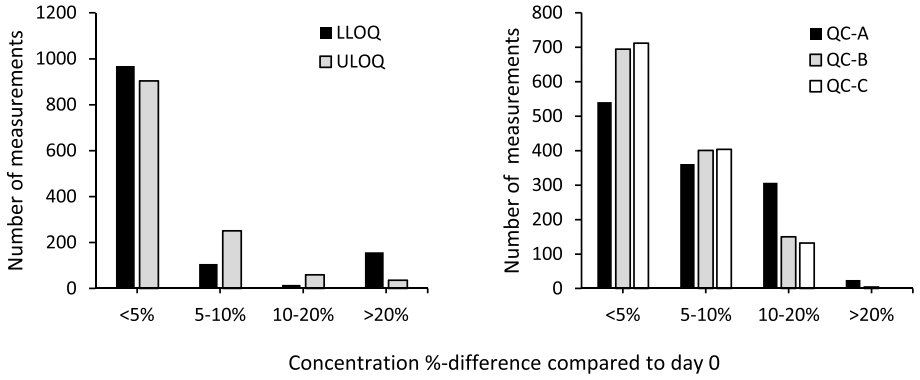

Figure 3. Distribution of the percent differences in the LLOQ, ULOQ, and QC sample concentrations for each of the 125 peptides for days 1 through 20 compared to day 0. LLOQ and ULOQ values for each peptide for days 1 and above were compared with the LLOQ and ULOQ values for the same peptide at time point 0, and the % differences were determined. Similarly, the average QC sample concentration values obtained for each peptide at levels A, B, and C for time point 0 were compared to the values obtained for day 1 and above (three replicates per QC sample, per time point). The number of measurements with % differences of <5%, between 5 and 10%, between 10 and 20%, and above 20% difference are shown. NAT peptide mixture levels for standards H, G, F, E, D, C, B, and A, were 100, 40, 16, 4, 2,0.5, 0.25, and 0.1× LLOQ/μL, respectively.

Acquity UPLC I-Class coupled to the Xevo TQ-XS tandem-quadrupole MS system in Iceland, as described below.

LC Separation and MS Analysis—Agilent System, Montreal, Canada. The digested plasma samples were resolubilized in aqueous 0.1% FA to give a final estimated concentration of 1 μg/μL total digested protein. Then, 10 μL (10 μg on column) of each plasma digest sample, QC sample, and calibration curve standard were injected into a Zorbax Eclipse Plus RP-UHPLC column (2.1 × 150 mm, 1.8 μm particle diameter; Agilent) within an Agilent 1290 Infinity II system at 50 ° C, interfaced to an Agilent 6495B MS for LC/MRM-MS analysis. The mobile phases used were 0.1% FA in LC−MS-grade water and 0.1% FA in LC−MS-grade ACN. Peptides were separated at a flow rate of 0.4 mL/min over a 52.6 min run, using a multistep LC gradient. The gradient was set up to start at 2% organic mobile phase, increase to 7% at 2 min, then to 30% at 50 min, 80% at 50.1 min, maintained at 80% until 52.5 min, and then reset back to 2% at 52.6 min. A postgradient column re-equilibration of 4 min was used after the analysis of each plasma sample, QC sample, and calibration curve standard.

The Agilent 6495B QqQ instrument was operated in the positive-ion mode, and dynamic MRM data were acquired at 3.5 kV and 300 V for the capillary voltage and nozzle voltage, respectively. The sheath gas flow was set to 11 L/min at a temperature of 250 °C, and the drying gas flow was set to 15 L/min at a temperature of 150 °C, with the nebulizer gas pressure at 30 psi. The collision cell accelerator voltage was set to 5 V, and the unit mass resolution was used in the first and third quadrupole mass analyzers. The high-energy dynode (HED) multiplier was set to −20 kV for improved ion detection efficiency and signal-to-noise ratios. The total cycle time was set to 700 ms with 90−120 s retention time windows. A single transition per peptide target was monitored.

LC Separation and MS Analysis—Waters System, Reykjavik, Iceland. Digested plasma samples, QC samples, and calibration curve standards were rehydrated, as mentioned above, and 10 μL of each was injected into an Acquity UPLC Peptide BEH C18 column (2.1 × 150 mm, 1.7 μm particle diameter; Waters) on a Waters Acquity UPLC I-Class system at 50 °C for LC/MRM-MS analysis. The mobile-phase composition was the same as mentioned above, and the peptides were separated at a flow rate of 0.4 mL/min over a 56.0 min run, using a multistep LC gradient. The gradient was set up to start at 2% organic mobile phase, increase to 7% at 2 min, then to 30% at 50 min, 45% at 53 min, 80% at 53.5 min, maintained at 80% until 55.5 min, and then reset back to 2% at 56.0 min. Column re-equilibration for 4 min was carried out after each analysis, as mentioned above. MS analysis was performed on a Xevo TQ-XS tandem-quadrupole mass spectrometer operated in the positive-ion mode. MRM data were acquired at 3.0 kV capillary voltage and a cone voltage of 28.0 V. The desolvation gas flow was set to 1200 L/h at a temperature of 650 °C, and the cone gas flow was set to 150 L/h, with the nebulizer gas pressure at 100 psi. A single transition per peptide target was monitored.

Data Analysis

Visual examination of the LC/MRM-MS data obtained was performed using the Skyline Quantitative Analysis software (version 20.1.0.76, University of Washington).[13] The chromatographic peaks for NAT and SIS peptides in plasma samples, calibration curves, and QC samples were assessed manually for peak shape and accurate integration. Calibration curves were generated using $1/x^2$-weighted linear regression and were used to calculate peptide concentrations in the samples as fmol/μL of plasma. Additional data analysis and visualization was performed using R (v3.5.3), Illustrator CS6, Microsoft Power Point, and Microsoft Excel. Each protein was quantified using a single tryptic surrogate peptide.

Standard and QC Acceptance Criteria. For the calibration curve standards and QC samples to be considered acceptable, the quantitative results were required to fall within ±20% of their theoretical concentrations; otherwise, they were rejected. Furthermore, the calibration curves for all 11 time points investigated were required to contain at least five points with concentration accuracies within ±20%. At least 90 and 66% of all individual calibration curve standards and QC samples, respectively, were required to fall within these criteria for the experiment to be considered successful.

RESULTS AND DISCUSSION

Performance of Calibration Curves and QC Samples

A total of 11 sets of calibration curves were generated, one set per time point, and the precision and accuracy were assessed. An overview of all data points collected, including the LLOQs,

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J. Proteome Res. 2021, 20, 4292−4302

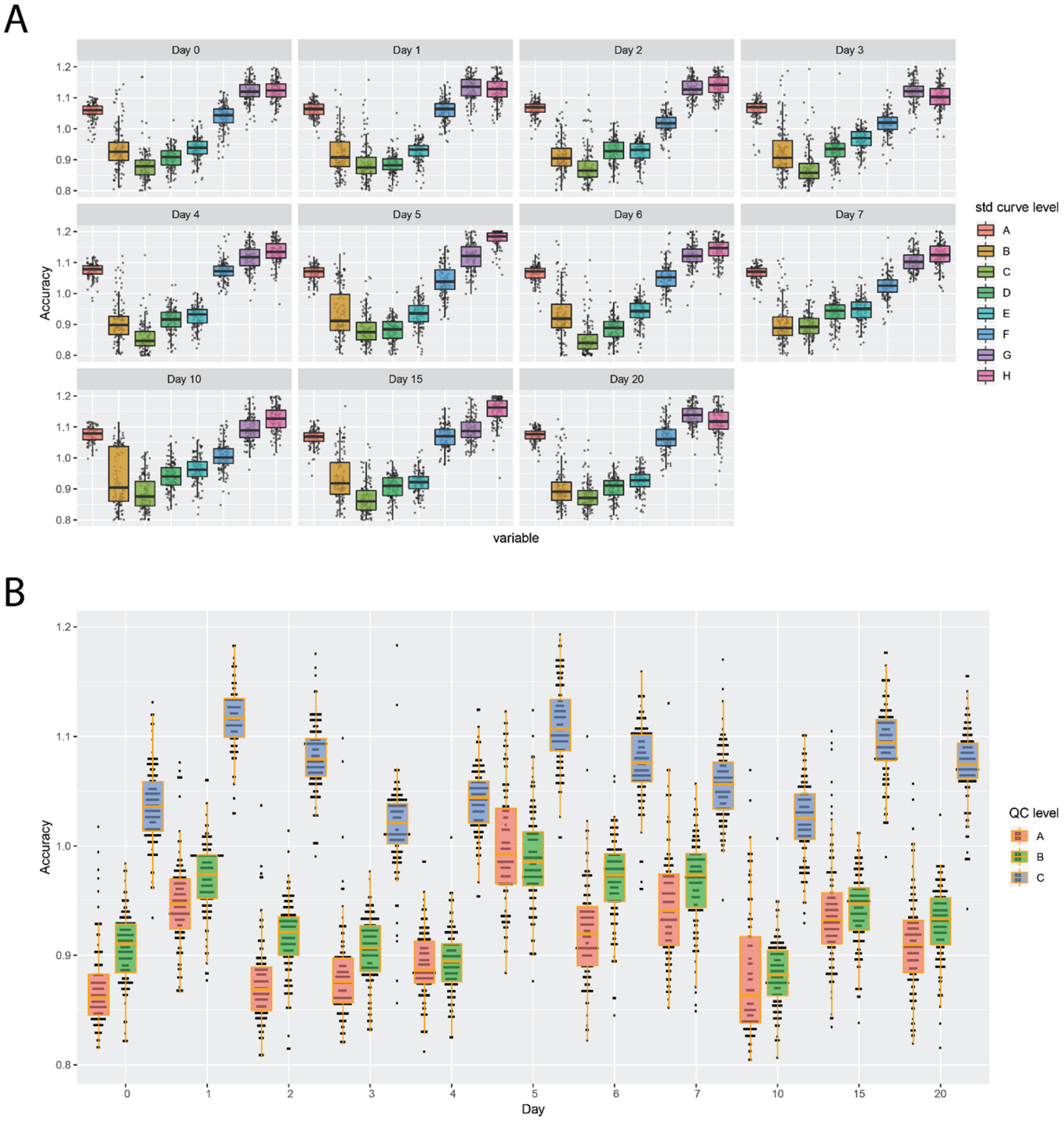

Figure 4. Accuracy ranges determined for (A) calibration curve points as well as (B) QC sample levels A, B, and C generated using the NAT peptide mixtures stored at room temperature for 0 to 7, 10, 15, and 20 days.

the ULOQs, as well as the QC-A, QC-B, and QC-C concentration values, is shown in Figure 2. The concentration range across all peptides analyzed covered approximately five orders of magnitude. The concentration values obtained across the various days were very consistent (Figure 3), indicating that the storage of the NAT peptide mixtures at room temperature for up to 20 days did not impact the quantitative results.

Accuracy values for the points on the calibration curve and QC sample levels A, B, and C generated using the NAT mixtures stored between 0 and 20 days at room temperature are shown in Figure 4. The majority of data points fell within 80 and 120%, indicating that the accuracy was not influenced by the prolonged storage of the NAT peptide mixtures at room temperature. This is further supported by the pattern observed in Figure 4A. Interestingly, the accuracies for QC-C (the QC sample with the highest concentration) were consistently slightly above 100%, whereas QC-A, with the exception of day 5, was consistently below 100% (Figure 4B). This result could be due to the weighting strategy of the calibration curves and could possibly be improved by choosing an alternative or an iterative weighting approach. However, given that the peptide

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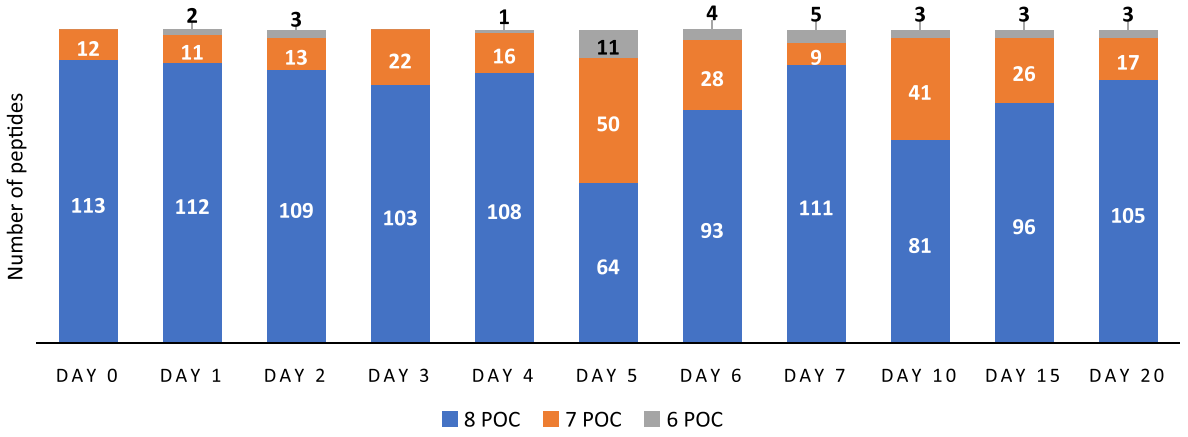

Figure 5. POC, out of 8, for the calibration curves generated for the 125 peptides. Each point on the calibration curve that was within ±20% of the theoretical concentration was included as a POC.

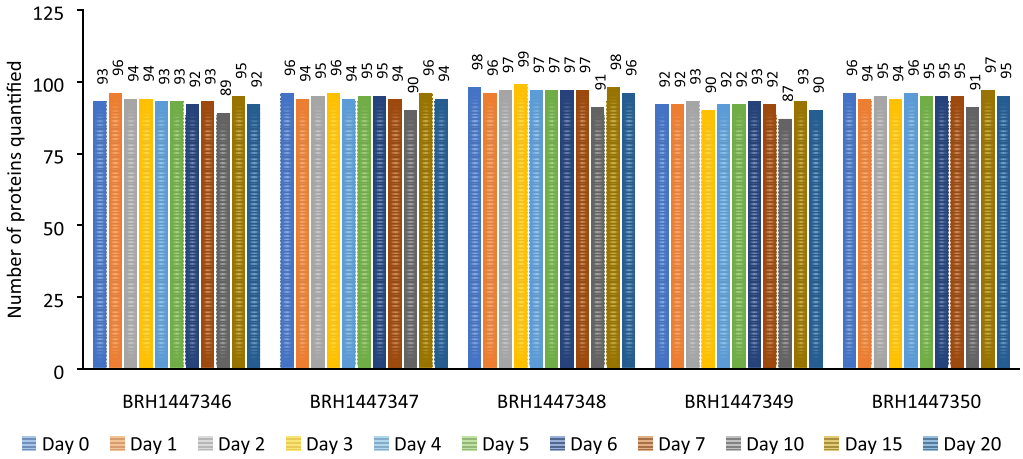

Figure 6. Number of proteins with quantifiable endogenous concentrations, as well as standard curves and QC samples within the acceptance criteria in five plasma samples between 0 and 20 days.

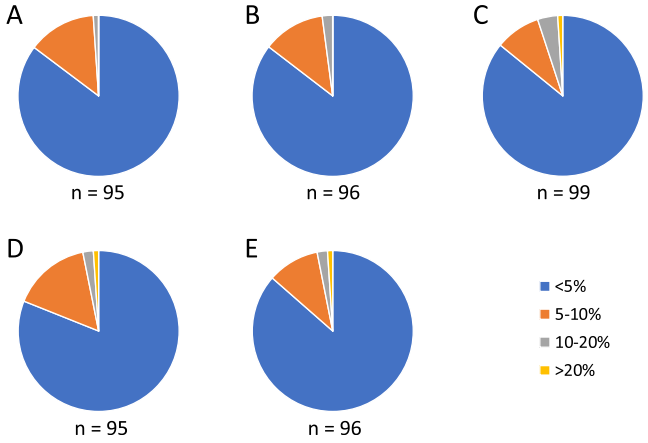

Figure 7. Distribution of %CVs calculated for all sample protein concentrations determined on all 11 time points for the five human plasma samples analyzed. (A) BRH1447346, (B) BRH1447347, (C) BRH1447348, (D) BRH1447349, and (E) BRH1447350.

accuracies were well within the 100 ± 20% range, the analytical performance of the assay was considered to be sufficient.

Most of the QC samples fell within the acceptance criteria mentioned above (at least 66% of all QC samples within ±20% of the theoretical concentration). Indeed, as visualized in Supporting Information Figure S1, a minimum of ≥89% of the 375 QC samples (125 peptides × 3 replicates) for each QC sample concentration level per day met the stringent acceptance criteria outlined above. It is important to note that even on day 20, the number of peptide QC samples that met our acceptance criteria was not significantly different from previous days, suggesting that exposing the lyophilized peptides to room temperature in a properly sealed container and in the presence of a sufficient amount of desiccant material for 20 days had no significant impact on MS-based quantitation.

The calibration curves for all 125 peptides were also evaluated in accordance with the criteria described above. It was found that most individual calibration curve standards (97.2%) were within ±20% of the theoretical values. Moreover, all standard curves consisted of at least 6 out of 8 standard levels (5/8 required, as per criteria). The LLOQ and ULOQ of each curve for each time point were determined by the lowest and highest accepted calibration curve standards, typically A and H, respectively.

Figure 5 shows the proportion of standard curves with 6, 7, and 8 points on the curve (POC) for all room-temperature incubation time points assessed. Across all days, the quantitation of the 125 peptides resulted in calibration curves with 8 POC (100 peptides on average), followed by 7 POC (22 peptides on average), and rarely 6 POC (3 peptides on

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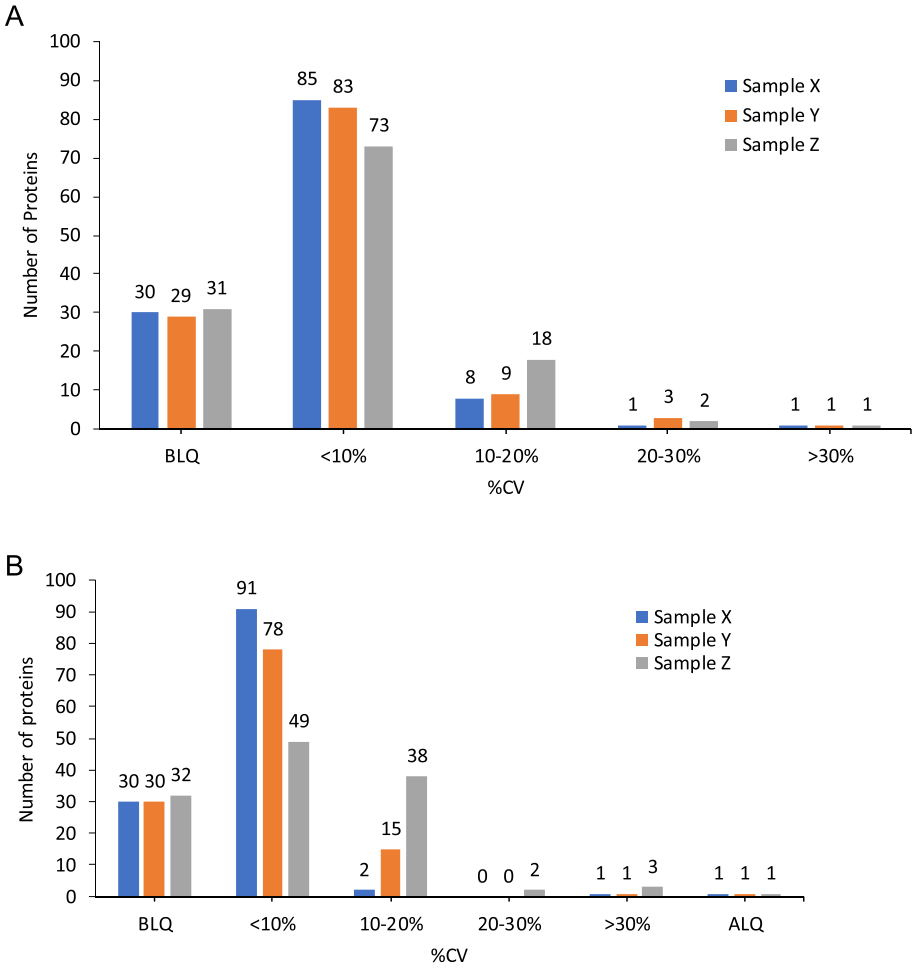

Figure 8. Overview of %CV values for protein concentrations quantified in four replicates of three different human plasma samples using an (A) Agilent 6495B (MRM Proteomics Inc., Canada) and a (B) Waters Acquity UPLC I-Class/Xevo TQ-XS system (University of Iceland, Iceland).

average). Thus, all 125 of the peptides passed the experimental criteria, and none of the curves were rejected on any experimental day. This highlights the consistent linear performance of these peptides in MRM analysis. Additionally, days 0 and 3 exhibited calibration curves with exclusively 7 or 8 POC. While up to day 4, there appears to be a trend with a consistent number of 8 POC for 103 to 113 peptides; this trend ends on day 5, where only 64/125 peptides achieved 8 POC.

However, as shown in Supporting Information Figure S2, the number of acceptable calibration curve standards increases again on day 6 and remains steady until experimental day 20. It, therefore, appears unlikely that the observed drop in performance was actually due to the degradation of the target peptides and was instead probably a random event.

The higher proportion of peptides with a lower number of POC observed on day 5 can be explained by an increase in the number of rejected standards A and H, leading to a greater number of peptide standard curves with 7 and even 6 points. This apparent decrease in standard points passing acceptance criteria on day 5 is most likely a consequence of a sudden decrease in the instrument performance. Indeed, while the calibration curve data for day 5 were being acquired, one of the pumps in our Agilent system showed signs of decreased performance and required repair and, ultimately, replacement. This affected the LLOQs and ULOQs measured on that particular day. Even though a decline in the instrument performance was observed, the data from that day were included because the results support the robustness of the MRM assays. This further shows that a lyophilized peptide mixture, as described here, is a suitable way to track the LC−MS instrument performance over time.

Moreover, while the instrument awaited a service engineer to repair the failed pump, the remaining samples were stored already rehydrated at −80 °C for a few days before analysis, further highlighting the strength and reliability of MRM technology, and specifically the use of SIS peptide standards for accurate and reliable protein concentration determination.

Protein Quantitation in Five Different Human Plasma Samples

The 11 sets of calibration curves were used to calculate the 125 protein concentrations from five different human plasma samples. Despite the different number of POC observed at different time points at room temperature (Figure 5), and including the lower numbers observed on day 5, the number of proteins quantified on each day was very consistent and ranged from 87 to 99 per day (Figure 6), resulting in percent coefficients of variation (%CVs) of <2% across the 11 test days for the number of proteins quantified for each sample. A total of 82 proteins were quantifiable in all the samples at all time points. As expected, no protein in any sample was found to be

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J. Proteome Res. 2021, 20, 4292−4302

Table 1. Proteins Determined to Be BLQ in the Three Human Plasma Samples Analyzed in Both Laboratories[a]

peptide	protein	BLQ in all three plasma samples	
		Canada (Agilent)	Iceland (Waters)
AHVNSLGENLK	interleukin-10	X	X
DLQNFLK	protein S100-A9	X	X
ETSNFGFSLLR	protein Z-dependent protease inhibitor	X	X
EYLIAGK	metalloproteinase inhibitor 2	X	-
FTTDLDSPR	tenascin	X	X
FYYNSVIGK	tissue factor pathway inhibitor (isoform 1)	X	X
GFVEPDHYVVVGAQR	transferrin receptor protein 1	X	X
GFYFNKPTGYGSSSR	insulin-like growth factor I	X	X
GHQAFDVGQPR	cation-independent mannose-6-phosphate receptor	-	X
GLLSGWAR	vitamin K-dependent protein Z	X	X
ILISGLEPSTPYR	tenascin-X | Putative tenascin-XA	X	X
INENTGSVSVTR	cadherin-13	X	X
ITPSYVAFTPEGER	78 kDa glucose-regulated protein	X	X
KPQDFLEELK	thrombospondin-4	X	X
LHPTHYSIR	coagulation factor VIII	X	X
LIDQYGTHYLQSGSLGGEYR	complement component C7	X	X
LLGIETPLPK	Intercellular adhesion molecule 1	X	X
LVNVVLGAHNVR	myeloblastin	X	X
SALVLQYLR	coagulation factor IX	X	X
SDLAVPSELALLK	galectin-3-binding protein	X	X
SEAYNTFSER	coagulation factor V	X	X
SLLEGEGSSGGGGR	keratin-type I cytoskeletal 10	X	X
SPLNDFQVLR	hepatocyte growth factor-like protein	X	X
TGVITSPDFPNPYPK	mannan-binding lectin serine protease 1	-	X
TLAFPLTIR	endothelial protein C receptor	X	X
TPAYYPNAGLIK	apolipoprotein(a)	X	X
VFQQVAQASK	plasminogen activator inhibitor 1	X	X
VVPGEEEQK	tissue-type plasminogen activator	X	X
WPEPVFGR	mannan-binding lectin serine protease 2	X	X
YEELQVTVGR	keratin-type II cytoskeletal 2 epidermal	X	X

[a]X indicates BLQ; - indicates Not BLQ.

Table 2. Percent Difference Ranges between the Plasma Concentrations of Each Protein Quantified in the Three Samples in Iceland and Canada

% difference ranges	number of proteins in each % difference range		
	sample X	sample Y	sample Z
Outside of Linear Range			
BLQ	29	28	27
BLQ Canada	2	2	2
BLQ Iceland	3	2	3
ALQ Canada			
ALQ Iceland	1	1	1
total outside linear range	35	33	33
Within Linear Range			
<10%	44	28	50
10−20%	22	30	20
20−30%	9	22	4
>30%	15	12	18
total within linear range	90	92	92
total number of proteins	125	125	125

above the ULOQ, and only 22 proteins were below their LLOQs in all samples across all time points.

Thus, the storage of lyophilized NAT peptide mixtures for up to 20 days at room temperature did not lead to significant changes in the measured concentrations of proteins in the plasma samples tested. Additionally, using calibration curves, where only 6 or 7 standards (rather than 8) passed the acceptance criteria (e.g., on days 5 and 10), did not seem to affect quantitation, even when the instrument performance was less than ideal, as was the case for day 5.

Next, to determine the precision of endogenous protein concentrations, we calculated the %CVs for the concentration of each protein for the days where there was a quantifiable result. Only proteins with quantifiable results on at least three of the 11 days were considered. The distribution of these CVs is shown in Figure 7.

The majority of proteins quantified (≥77%) resulted in CVs <5% for each sample, followed by a CV of 5−10%, and rarely 10−20%. Only two of the quantified proteins showed %CVs of >20%—protein AMBP in sample BRH1447348 and cation-independent mannose-6-phosphate receptor in samples BRH1447349 and BRH1447350. The high CVs did not appear to be correlated to any particular physicochemical peptide properties or specific amino-acid residues. These results demonstrate the high precision of the data despite the light peptide mixtures having been stored for up to 2 weeks at room temperature prior to sample preparation.

Comparison of Protein Concentrations Determined in Iceland vs Canada

Three different plasma samples were sourced and analyzed in Iceland using the light and heavy peptide mixtures for 125

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J. Proteome Res. 2021, 20, 4292−4302

targets shipped without dry ice from Canada. Four aliquots of each plasma sample were digested and further processed as described in the Methods section above. After digestion and cleanup, each sample aliquot was analyzed in singlicate on a Waters Acquity UPLC I-Class/Xevo TQ-XS system, and the data were evaluated using Skyline (version 20.1.0.76, University of Washington). For assay comparison, aliquots of the three raw plasma samples obtained in Iceland were shipped to Canada on dry ice for analysis using an Agilent 1290 Infinity II UPLC/6495B QqQ, followed by Skyline (version 20.1.0.76, University of Washington) data processing. Heavy- and light-peptide-mixture standards for the 125 target proteins that had been maintained at −80 °C were used as standards for the analysis in Canada. Upon calculating protein concentrations obtained at both sites, %CVs were calculated across sample replicates measured on the Agilent 1290 Infinity II UPLC/6495B QqQ (Figure 8A) and the Waters Acquity UPLC I-Class/Xevo TQ-XS setup (Figure 8B). The protein concentrations determined by both sites were found to be comparable, with only 29−32 proteins being below the limit of quantitation (BLQ). Table 1 shows the 27 proteins that were BLQ in all three samples in both Iceland and Canada, the two proteins that were BLQ in Canada only, and the one protein that was BLQ in Iceland only. The data demonstrate similar performances of both the LC−MS systems and the lyophilized heavy and light peptide mixtures for the absolute quantitation of human plasma proteins. This is despite a 5-day-long transport of the heavy and light peptide mixtures without dry ice, used in Iceland.

The majority of the %CVs between replicate sample preparations were found to be <10%, and only 1−3 proteins showed CVs >30% (Figure 8). Similar to the results summarized in Figure 7, the high-CV peptides in Figure 8 showed no clear trend between the high CVs and specific physicochemical characteristics. Interestingly, serum albumin (LVNEVTEFAK) was found to be above the level of quantitation (ALQ) in the analysis carried out on the Waters system. Table 2 gives an overview of the proteins outside and within the linear ranges, as well as the percent differences between average protein concentrations determined in Canada and Iceland for four replicates of the three plasma samples evaluated. Results show that, in the three samples obtained in Iceland, 90−92 of the proteins quantified in Canada and Iceland were within the linear ranges, and only 33−35 proteins were outside the linear ranges. Among these, 80−87% of proteins quantified showed <30% difference between the concentrations determined on the two different instrumental setups. Concentration differences higher than 30% can potentially be explained by interferences observed in one LC/MS system versus the other. A detailed overview of these differences is shown in Supporting Information Table S4.

CONCLUSIONS

Aliquots of a 125-peptide mixture for the quantitative analysis of 125 human plasma proteins by MRM were stored at room temperature with controlled humidity for extended periods of time to assess the reliability of protein quantitation after storage at above-freezing temperatures. Five commercial human plasma samples were measured using 11 standard curves at different room-temperature-incubation time points of up to 20 days, and the results showed strong %CVs, most of which were below 5%. Overall, no decrease in the quantitative performance over time was observed, as demonstrated by high precision and maintained sensitivity between 0 and 20 days. Three additional human plasma samples were analyzed at two different sites, one using light and heavy peptide standard mixtures shipped without dry ice and the other using never-thawed standards. A comparison of the results from both analyses showed that more than 80% of the quantifiable peptides exhibited concentration differences of less than 30% between the values obtained in Iceland, using light and heavy peptide mixtures shipped lyophilized, without dry ice and with desiccants, and the results obtained in Canada with fresh, never-thawed light and heavy lyophilized mixtures. Overall, the results suggest that lyophilized peptide mixtures can be shipped without dry ice, as long as the humidity is controlled.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00249.

MRM peptides used for the quantitation of 125 human plasma proteins; lot numbers of commercial human plasma analyzed; concentration range details for the standard curves generated for each peptide used; overview of percent differences of 125 peptides quantified from three plasma samples in Canada and Iceland on two instrumental setups; percentage of QC-A, -B, and -C sample replicates meeting criteria for each of the 125 surrogate peptides measured from day 0 to 7, 10, 15, and 20; percentage of accepted calibration curve standards and rejected calibration curve standards for each of the 11 curves generated after incubation at room temperature from 0−7, 10, 15, and 20 days (PDF)

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Notes

The authors declare the following competing financial interest(s): CHB is the CSO of MRM Proteomics, Inc. CG, RP, and SPB are staff members at MRM Proteomics, Inc.

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■ REFERENCES

(1) Gordy, D.; Tashjian, R. S.; Lee, H.; Movassaghi, M.; Yong, W. H. Domestic and International Shipping of Biospecimens. *Methods Mol. Biol.* **2019**, *1897*, 433−443.

(2) Auer, H.; Mobley, J. A.; Ayers, L. W.; Bowen, J.; Chuaqui, R. F.; Johnson, L. A.; Livolsi, V. A.; Lubensky, I. A.; McGarvey, D.; Monovich, L. C.; Moskaluk, C. A.; Rumpel, C. A.; Sexton, K. C.; Washington, M. K.; Wiles, K. R.; Grizzle, W. E.; Ramirez, N. C. The effects of frozen tissue storage conditions on the integrity of RNA and protein. *Biotech. Histochem.* **2014**, *89*, 518−528.

(3) Australia Post. *Dangerous and prohibited goods and packaging guide*; Melbourne VIC, 2020.

(4) Houchin, M. L.; Heppert, K.; Topp, E. M. Deamidation, acylation and proteolysis of a model peptide in PLGA films. *J. Controlled Release* **2006**, *112*, 111−119.

(5) Han, J.; Higgins, R.; Lim, M. D.; Lin, K.; Yang, J.; Borchers, C. H. Short-Term Stabilities of 21 Amino Acids in Dried Blood Spots. *Clin. Chem.* **2018**, *64*, 400−402.

(6) Bettinger, J. Q.; Welle, K. A.; Hryhorenko, J. R.; Ghaemmaghami, S. Quantitative Analysis of in Vivo Methionine Oxidation of the Human Proteome. *J. Proteome Res.* **2020**, *19*, 624−633.

(7) Brennan, T. V.; Clarke, S. Spontaneous degradation of polypeptides at aspartyl and asparaginyl residues: effects of the solvent dielectric. *Protein Sci.* **1993**, *2*, 331−338.

(8) Geiger, T.; Clarke, S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* **1987**, *262*, 785−794.

(9) Steffen, P.; Krisp, C.; Yi, W.; Yang, P.; Molloy, M. P.; Schlüter, H. Multi-laboratory analysis of the variability of shipped samples for proteomics following non-cooled international transport. *Anal. Biochem.* **2018**, *548*, 60−65.

(10) Gaither, C.; Popp, R.; Mohammed, Y.; Borchers, C. H. Determination of the concentration range for 267 proteins from 21 lots of commercial human plasma using highly multiplexed multiple reaction monitoring mass spectrometry. *Analyst* **2020**, *145*, 3634−3644.

(11) Kuzyk, M. A.; Smith, D.; Yang, J.; Cross, T. J.; Jackson, A. M.; Hardie, D. B.; Anderson, N. L.; Borchers, C. H. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* **2009**, *8*, 1860−1877.

(12) CPTAC Assay Characterization Guidance Document. https://proteomics.cancer.gov/assay-portal/about/assay-characterization-guidance-documents, Access date: Nov. 4, 2015.

(13) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **2010**, *26*, 966−968.