Multiple Reaction Monitoring-Mass Spectrometry Enables Robust Quantitation of Plasma Proteins Regardless of Whole Blood Processing Delays That May Occur in the Clinic

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In Brief
In the clinic, delays between blood collection and plasma generation are often unavoidable, possibly impacting intact protein-assay measurements, such as ELISA. Here we investigated the impact of plasma processing delays (0 to 40 h) on peptide-centric protein quantitation via validated LC/MRM-MS assays. From 159 LC/MRM-MS assays, 139 were ‘stable’ (RSD < 20%), 14 ‘semistable’ (RSD 20–30%), and 6 ‘unstable’ (RSD > 30%), demonstrating robustness and thus the potential for plasma-protein quantitation by validated LC/MRM-MS assays in a clinical setting.

Highlights
- Delays in whole blood processing often cannot be avoided in the clinic.
- These delays might affect measurements by intact protein assays such as ELISA.
- The impact on LC/MRM was evaluated using validated assays to quantify 270 proteins.
- >95% of the measured concentrations had RSDs <30% between delays of 0 to 40 h.
- Protein quantitation by LC/MRM-MS is robust against pitfalls in the clinical setting.

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Multiple Reaction Monitoring-Mass Spectrometry Enables Robust Quantitation of Plasma Proteins Regardless of Whole Blood Processing Delays That May Occur in the Clinic

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Plasma is an important biofluid for clinical research and diagnostics. In the clinic, unpredictable delays—from minutes to hours—between blood collection and plasma generation are often unavoidable. These delays can potentially lead to protein degradation and modification and might considerably affect intact protein measurement methods such as sandwich enzyme-linked immunosorbent assays that bind proteins on two epitopes to increase specificity, thus requiring largely intact protein structures. Here, we investigated, using multiple reaction monitoring mass spectrometry (MRM-MS), how delays in plasma processing affect peptide-centric “bottom-up” proteomics. We used validated assays for proteotypic peptide surrogates of 270 human proteins to analyze plasma generated after whole blood had been kept at room temperature from 0 to 40 h to mimic delays that occur in the clinic. Moreover, we evaluated the impact of different plasma-thawing conditions on MRM-based plasma protein quantitation. We demonstrate that >90% of protein concentration measurements were unaffected by the thawing procedure and by up to 40-h delayed plasma generation, reflected by relative standard deviations (RSDs) of <30%. Of the 159 MRM assays that yielded quantitative results in 60% of the measured time points, 139 enabled a stable protein quantitation (RSD <20%), 14 showed a slight variation (RSD 20–30%), and 6 appeared unstable/irreproducible (RSD > 30%). These results demonstrate the high robustness and thus the potential for MRM-based plasma-protein quantitation to be used in a clinical setting. In contrast to enzyme-linked immunosorbent assay, peptide-based MRM assays do not require intact three-dimensional protein structures for an accurate and precise quantitation of protein concentrations in the original sample.

Laboratory test results are an essential part of the decision-making process in clinical diagnostics and treatment therapies (1). Because blood carries oxygen and nutrients throughout the body, it is in close contact with all tissues and organs, making it an attractive biofluid for minimally invasive health monitoring and diagnostics. Indeed, blood components can be used to evaluate a patient’s health status, and routine medical tests generally involve blood collection by phlebotomy to assess the presence, absence, upregulation, or downregulation of specific disease-related biomarkers (2).

In the past decade, a number of proteomic studies have analyzed plasma samples through discovery proteomics, aiming for the relative quantitation of a large number of proteins (3–5). These studies are often followed by targeted multiple reaction monitoring (MRM) for validation of the proposed biomarkers by the absolute quantitation of specific plasma proteins (6–9). Clinical proteomic studies are strongly dependent on having access to a sufficient number of samples, especially during the discovery stage, to detect relative differences in clinical samples for subsequent validation (10). Biobanks are an important resource for the collection and storage of relevant clinical samples but, despite efforts toward the standardization of sample collection techniques to minimize technical variation (10), unexpected events can hinder the proper implementation of standard operating procedures. For instance, a high intake of patients during peak hours or extraordinary events, or the presence of overworked staff or understaffed clinics and hospitals, can lead to greater variations in sample handling, sample processing delays, and inadequate sample storage for prolonged periods of time after collection. Pandemics, such as the recent COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2, is an example of such a situation in

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which an already overworked and stressed health sector takes on more patients and more sample collection for diagnostics than would be handled under regular circumstances (11).

Technical variability in sample handling and collection has been shown to cause differences in proteomic and metabolomic profiles (12, 13). Variations in sampling procedures, such as the type of blood collection tubes and anticoagulants used, clotting and storage time before centrifugation, and degree of hemolysis, have all been shown to affect plasma protein concentration measurements (14, 15). Additionally, blood kept in collection vials undergoes pre-analytical changes and certain components have been shown to degrade rapidly (16). If ideal laboratory practices were followed, blood samples would be processed immediately, but that is not always possible in the clinic. The process of freezing and thawing whole blood leads to hemolysis and adds a freeze-thaw cycle which could further add to analytical variability (17). Therefore, the two most widely utilized options when immediate processing is not possible are to maintain the blood either at 4 °C or at room temperature, followed by plasma generation, and subsequent storage below −20 °C (14). Platelets have been shown to behave differently and undergo aggregation under cooler temperatures (compared to when blood is held at room temperature, where no aggregation occurs (18)), leading to the secretion of specific subsets of proteins and metabolites from the platelet proteome and metabolome into the blood (19, 20). As a consequence of the above-mentioned points, methods and assays that solely rely on the handling and measurement of intact proteins, such as ELISA, can potentially be negatively impacted by the nonideal sample handling that might occur in the clinic. In contrast to intact protein quantitation methods, liquid chromatography (LC)/MRM targets selected proteotypic peptides generated upon proteolytic digestion as surrogates for protein concentrations. Thus, we hypothesized that LC/MRM might be less affected by delays in whole blood processing and would still reflect the original plasma protein concentrations with high accuracy and precision. To verify this, we utilized assays that had been validated according to the Clinical Proteomic Tumor Analysis Consortium’s (CPTAC) Tier 2 criteria (21) for the “absolute” quantitation of 270 protein targets by LC/MRM mass spectrometry (LC/MRM-MS; see Fig. 1) using stable isotope-labeled standard (SIS) peptides as internal standards.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents and Labware—Phosphate buffered saline tablets, Trizma preset crystals (pH 8.0), urea, DTT, iodoacetamide, and bovine serum albumin (BSA) were purchased from Sigma Aldrich. Axygen 1.1 ml deep 96-well plates and 1 ml 96-well plates from Waters were used. Protein LoBind tubes and LoBind 96-well PCR 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, and acetonitrile (ACN), all LC-MS grade, were purchased from Fisher Scientific or Sigma-Aldrich.

**Peptide Mixtures**—The peptide standard mixtures used for this project were from MRM Proteomics Inc.’s PeptiQuant 270-protein human plasma MRM assay kit. The kit contains a lyophilized native (natural, light [NAT]) and a SIS peptide mixture, each consisting of 274 peptides, as well as trypsin and BSA. The synthetic proteotypic peptides in the two mixtures serve as molecular surrogates for 270 human plasma proteins (the peptide sequences and target proteins are shown in supplemental Table S1). The selection process for these surrogate peptides involved stringent rules and a required set of characteristics, as has been described previously (22). Briefly, surrogate tryptic peptides for the 270 proteins had been carefully selected in silico using PeptidePicker (23). The Universal Protein Resource (UniProt) (24) was consulted to ensure uniqueness, absence of modifications and variants as much as possible. The synthetic peptides had been tested for detectability when spiked into human plasma, and precursor ions and transitions for each peptide were selected and ionization conditions were optimized empirically using Agilent MassHunter Optimizer (https://www.agilent.com/cs/library/emanuals/public/K3793-90008_Optimizer_QuickStart.pdf).

As described previously (25), peptide purities and accurate concentrations of the kit components had been determined by capillary zone electrophoresis and amino acid analysis (25). Limits of detection, lower limits of quantitation (LLOQ), upper limits of quantitation and precision had previously been established following the CPTAC’s guidelines for assay development (https://proteomics.cancer.gov/assay-portal/about/assay-characterization-guidance-documenta) (26), which are available on the CPTAC Assay portal website (https://proteomics.cancer.gov/assay-portal). The concentrations of the standards used to generate the calibration curves for each peptide covered five orders of magnitude. Peptide mixture dilutions were prepared in Eppendorf Protein LoBind tubes.

**Human Blood Samples**—Commercially available human whole blood samples from five different individuals were purchased from BioIVT, which “conducts its human research activities in accordance with regulations surrounding human research subject safety and protection which include ethical principles that have their origin in the Declaration of Helsinki and are consistent with Good Clinical Practice” (https://www.vertastk.co.jp/products/BioIVT%20Ethical%20Procurement%20of%20Human%20and%20Animal%20Biological%20Specimens.pdf). The BioIVT samples had been collected in Becton, Dickinson and Company whole blood glass tubes with anticoagulant (acid citrate dextrose) (catalog number: 364608). Each sample was split into 20 aliquots, for a total of 100 replicates before shipment. The samples remained in transit for approximately 40 h and were maintained at 4 °C during transport, using icepacks and insulated packaging.

The whole blood samples were obtained from individual male and female donors of either Black or Hispanic ethnicity (see supplemental Table S2). Documentation provided by the supplier contained quality control (QC) details in compliance with U.S. Food and Drug Administration regulations. Whole blood donors had been tested with Food and Drug Administration Center for Biologics Evaluation and Research-licensed screening tests and showed negative results for hepatitis B virus surface antigen and nucleic acid test, HIV 1 and 2 AB (HIV-1 and -2 antibody) and nucleic acid test, Trypanosoma cruzi antibodies, HCV AB (hepatitis C virus antibody) and nucleic acid test, syphilis, West Nile virus, and Zika virus RNA. There were no reports on the health or disease status of the subjects, other than for the infectious diseases mentioned above.
Experimental Design and Rationale

Time Course Incubation of Five Whole Blood Lots at Room Temperature—Common situations in the clinical laboratory were mimicked by incubating five lots of commercially available whole blood for up to 40 h at room temperature (see experimental overview, Fig. 1). Thus, upon receipt, aliquots of each of the five sample lots were immediately subjected to a time course incubation at room temperature for 0, 1, 2, 3, 4, 5, 6, 16, 20, and 40 h. Two aliquots of each sample lot were incubated for each time point. After incubation, the aliquots were immediately centrifuged at 2000 × g for 10 min at room temperature to generate plasma. The plasma was then carefully removed without disturbing the red blood cell layer, transferred to clean Eppendorf LoBind tubes, and immediately frozen at −80 °C.

Quick and Overnight Thaw—We had recently obtained plasma samples from a local Biobank for a different research project and these samples were thawed overnight (O/N) in the refrigerator prior to aliquoting and transporting to our laboratory. Hence, the effect of two different thawing conditions on the plasma protein concentration values obtained via peptide-based assays was assessed in the current study.

Fig. 1. Experimental overview. Five whole blood samples collected in acid citrate dextrose (ACD) collection vials were aliquoted into two groups of ten aliquots each, for a total of 20 aliquots per sample (step 1). Next, one aliquot per group and sample was incubated at room temperature for 0, 1, 2, 3, 4, 5, 6, 17, 20, and 40 h (step 2). After each incubation period, the samples were immediately centrifuged to generate plasma (step 3). After centrifugation, the plasma samples were stored at −80 °C (step 4). After all of the samples were collected and frozen, all of the plasma samples from sample group 1 were thawed overnight for approximately 18 h at 4 °C (step 5), while plasma samples from group 2 were quickly thawed on ice for approximately 1 h. Finally, all samples were prepared following a bottom-up proteomics approach using stable isotope-labeled standard (SIS) peptides and were analyzed by multiple reaction monitoring (MRM) (step 6).
study. We compared our usual on-ice thawing followed by immediate processing procedure, to O/N thawing at 4 °C in a refrigerator—the procedure used by the Biobank. The plasma samples used for the current study were therefore thawed either on ice on the laboratory bench prior to immediate sample preparation and analysis (quick-thaw) or O/N for approximately 18 h at 4 °C in a refrigerator. Thawing was coordinated so that the tryptic digestion of all of the aliquots, both quick-thaw and O/N, was performed concurrently, as described below.

**Enzymatic Digestion of Plasma and Surrogate Matrix**

Tryptic digestion of the plasma aliquots and the BSA surrogate matrix was performed as described previously (27). Briefly, 10 μl of either BSA at 10 mg/ml or raw human plasma were reduced and denatured by addition of urea/DTT/Tris–HCl buffer at pH 8 to a final concentration of 6 M urea, 13 mM DTT, and 200 mM Tris–HCl. Samples were then incubated at 37 °C for 30 min. Proteins were then alkylated by adding iodoacetamide to produce a final concentration of 40 mM and the samples were incubated at room temperature in the dark for 30 min. Urea was diluted to <1 M by addition of 100 mM Tris buffer prior to adding TPCK-treated trypsin (Worthington) at a protein-to–enzyme ratio of 20:1. The digestion was quenched by acidifying the samples to a final concentration of 1.0% FA (pH ≤2), resulting in an estimated peptide mixture concentration of 1 μg/μl. The samples were kept on ice until the subsequent spiking of the SIS peptides and solid-phase extraction (SPE) clean-up.

**Calibration Curve Standards and QC Sample Preparation**

The digested-BSA-in-PBS-buffer surrogate matrix was used as background in which the calibration curve standards and the QC samples were prepared. The lyophilized 274 peptide-containing NAT matrix was reconstituted in 260 μl of 30% ACN/0.1% FA to yield a protein solution with a concentration of 100x LLOQ/μl. As described previously (27), the stock NAT solution was further serially diluted with 30% ACN/0.1% FA to yield eight concentrations: 100x, 40x, 16x, 4x, 2x, 0.5x, 0.25x, and 0.1x LLOQ/μl in order to generate a standard curve for each peptide in the mixture. In addition, three replicates of each QC sample were prepared at three different concentration levels by diluting the 100x LLOQ/μl NAT peptide stock. Thus, triplicate QC samples at final concentrations of 0.35x, 3.5x, and 35x LLOQ/μl, for QC-A, QC-B, and QC-C, respectively, were prepared and analyzed along with the plasma digest samples.

A lyophilized mixture containing the SIS-peptide versions of the 274 natural peptides of interest was rehydrated in 220 μl of 30% ACN/0.1% FA and then diluted to 10x LLOQ/μl with aqueous 0.1% FA to be used as a normalizer for all plasma samples, calibration curve standards, and QC samples.

Each eight-point calibration curve was prepared by combining 45 μl of the 10x LLOQ/μl SIS peptide mixture, 45 μl of the level-specific amount of light peptide mixture, and 45 μl of the digested surrogate matrix (BSA). Similarly, 55 μl of surrogate matrix, 55 μl of the SIS peptide mixture, and 55 μl of the corresponding level-specific amount of light peptide mixture were used to prepare QC samples A through C in triplicate. A 45-μl aliquot of each lot of human plasma digest from each time point, and each thawing condition was spiked with 45 μl of the SIS peptide mixture.

**Solid-Phase Extraction**

Solid-phase extraction sample processing was performed as described previously (27). Wells were conditioned with methanol and washed with aqueous 0.1% FA. Immediately after loading the plasma samples into the HLB μElution plate, the wells were washed with LC-MS grade water. Elution was done by adding a solution containing a high percentage of organic solvent (70% ACN/0.1% FA) to all wells.

After SPE clean-up, the resulting peptide eluates were dried in a SpeedVac vacuum concentrator and stored at −80 °C until mass spectrometry (MS) analysis. QC samples and calibration curve standards were not subjected to SPE cleanup, but were dried and then frozen. Plasma samples, calibration curve standards, and QC samples were then resolubilized and analyzed on an Agilent 1290 Infinity II UHPLC coupled to an Agilent 6495B triple quadrupole (QqQ) MS.

**Liquid Chromatography Separation and MS Analysis**

The dried peptides 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 was separated on a Zorbax Eclipse Plus RP-UHPLC column (2.1 × 150 mm, 1.8 μm particle diameter; Agilent) at 50 °C using an Agilent 1290 Infinity II system interfaced to an Agilent 6495B MS for LC/MRM-MS analysis. The mobile phases used were 0.1% FA in LC-MS grade water (A) and 0.1% FA in LC-MS grade ACN (B). The 56-min multi-step LC gradient at a flow rate of 0.4 ml/min was as follows. The gradient started at 2% B, ramped up to reach 7% B at 2 min, 30% B at 50 min, 45% B at 53 min, 80% B at 53.5 min, then held at 80% B until 55.5 min, and finally ramped down to 2% B at 56 min. A postgradient column re-equilibration of 4 min at 2% B was used after the analysis of each plasma sample, QC sample, and calibration curve standard.

The 6495B triple quadrupole instrument was operated in the positive ion mode. Multiple reaction monitoring data were acquired at 3.5 kV and 300 V 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 unit mass resolution was used in the first and third quadrupole mass analyzers. The high energy dynode multiplier was set to −20 kV for improved ion detection efficiency and signal-to-noise ratios. A single transition per peptide target was monitored over 700 ms cycles, and 90 s detection windows were used for the quantitative analysis.

To ensure proper performance of the system during the entire analysis, the QC samples were evaluated at different stages during the batch analysis. The injection order was as follows: QC samples from lowest to highest concentration (injection replicate 1), standard curve from lowest to highest concentration, QC samples from lowest to highest concentration (injection replicate 2), plasma samples thawed O/N, QC samples from lowest to highest concentration (injection replicate 3), plasma samples thawed quickly, and QC samples from lowest to highest concentration (injection replicate 4). One blank was injected in between the QC sample and the standard and plasma sample sections. In addition, the plasma samples were injected starting with sample lot 1 time point 0 h and moving consecutively to sample lot 1 time point 40 h before moving on to the next sample lot.

**Data Analysis**

Visual examination of the LC/MRM-MS data was performed using Skyline software (version 21.1.0.146, University of Washington) (26). The chromatographic peaks for the NAT, endogenous (originating from within the sample, END), and SIS peptides in the plasma samples, the calibration curves, and the QC samples were assessed manually for peak shapes, accurate integration, and consistent retention times to avoid potential interferences. Calibration curves were generated using 1/x2-weighted linear regression and used to calculate peptide concentrations in the samples as fmol/μl of plasma based on the light/heavy peak area ratios. Additional data analysis and visualization was performed using R (v3.6.2) utilizing the ‘mcr’ package (v1.2.2). Of the 270 target proteins, 266 were targeted based on a
single surrogate tryptic peptide, while four proteins (complement C4, insulin-like growth factor-binding protein complex acid labile subunit, tumor necrosis factor receptor superfamily member 1A, and vitamin K-dependent protein z) were covered by two surrogate tryptic peptides.

Perseus software (1.6.15.0) (29) was used to generate the heat-maps, after normalizing protein concentrations of the 1 h to 40 h time points to the corresponding 0 h time point for each plasma lot. The relative abundances obtained were filtered to keep only those proteins that were quantified in 80% of the 100 samples, and the abundances were then log2-transformed.

**Standard and QC Acceptance Criteria**

Calibration curve standards and QC samples were considered to be acceptable when the quantitative results were within ±20% of their theoretical concentrations. Values outside this range were rejected. In addition, the calibration curves were required to contain at least five of eight 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**

The 274 calibration curves generated, along with their respective QC samples, were evaluated according to the acceptance criteria described above. All of the standard curves met the criteria, with 97% and 95% of all standards and QC samples, respectively, falling within ±20% of their theoretical values (Table 1). The plasma protein concentrations in the five commercial sample lots for the ten time points evaluated were determined from the standard curves generated.

**Determination of Original Protein Concentration in Plasma Samples**

A total of 127 out of 274 peptides were quantifiable (i.e., above the LLOQ) across all 100 samples (i.e., all five whole blood lots, all time points, and quick versus O/N plasma thaws) analyzed. For the 0 h time points for all five sample lots, 144 peptides were quantified from the quick-thaw plasma samples and 143 peptides from the O/N-thaw samples. Relative standard deviations (RSDs) for all peptides with at least three quantifiable concentrations across the ten time points (0 h to 40 h) were calculated for each sample. The resulting RSD distributions for the quick-thaw plasma samples and the O/N-thaw plasma samples are shown in Figure 2A and Figure 2B, respectively. Most peptides quantified from the quick-thaw samples showed low RSDs, with 65%, 21%, 6%, and 6% of the peptides across all five sample lots having RSDs of <10%, 10 to 20%, 20 to 30%, and >30%, respectively (Fig. 2A). Each of the five samples showed a comparable trend in terms of RSD distribution.

The results for the O/N-thaw samples, however, showed an unexpectedly low number of peptides in the <10% RSD category and a higher-than-expected number of peptides in the 10 to 20% RSD group for samples 4 and 5, as shown in Figure 2B. To identify the reason for this discrepancy, the percent differences in the determined peptide concentrations for the quick-thaw and the O/N-thaw sample aliquots were calculated for all 1 h to 40 h time points compared to their 0 h time points (see Fig. 3). We found that, as expected, most protein concentrations determined were within a 30% difference when compared to their respective 0 h time point. However, the 6 h time point from sample 4 (Fig. 3I) and time points 1 h, 20 h, and 40 h from sample 5 (Fig. 3J) showed systematically higher percent-difference values than all other time points from all of the other samples. These particular four samples appeared to be clear outliers and skewed the RSD distribution results. After excluding these points from the data set, the recalculated RSDs (Fig. 2C) were in line with the expected distribution of RSDs, with 71%, 18%, 6%, and 4% of the peptides quantified having RSDs of <10%, 10 to 20%, 20 to 30%, and >30%, respectively. The RSD data, including median RSDs which ranged from 6.2% to 7.8% between samples, are available in the Supplemental Data file. Furthermore, the RSD data in the Supplemental Data file demonstrate that elevated RSDs are consistent across samples and independent of quick and O/N conditions, with a few exceptions as shown in the “HeatMap Diff Proteins” Excel sheet.

In addition, using two-tailed t-tests, we calculated p-values for the END levels, quantified across all five samples, comparing each time point with the corresponding 0 h time point (supplemental Fig. S1), as long as concentrations were above the LLOQ for at least 3 of the 5 samples per time point. The data showed a similar trend across all time points, with the vast majority of peptides showing no significant differences for both the ON (supplemental Fig. S1A) and QUICK (supplemental Fig. S1B) conditions, with 98.5% and 98.4% of all p-values being ≥0.05, respectively.

For each blood lot, the majority of protein concentrations appeared stable across all time points, as visualized by the

| QC/standard | QC level | QC-A | QC-B | QC-C | Standard level | A | B | C | D | E | F | G | H |
|-------------|----------|------|------|------|---------------|---|---|---|---|---|---|---|---|
| % Passed    | Mean     |      |      |      | % Passed      |   |   |   |   |   |   |   |   |
| QC-A        | QC-B     | QC-C |      |      | QC-A          |   |   |   |   |   |   |   |   |
normalized abundance values shown in yellow in the heatmaps for both the quick-thaw samples (Fig. 4A) and the O/N-thaw samples (Fig. 4B). Time point 6 h from sample 4, as well as time points 1 h, 20 h, and 40 h from sample 5 of the O/N-thaw samples had elevated numbers of proteins in the RSD range of 10 to 20% (see Figs. 2B and 3, I and J) and appeared as outliers in the heatmaps (Fig. 4B). The systematically low concentrations obtained for these four O/N-thaw samples could be explained by a pipetting error, where the wells for these specific time points received either less plasma or more of the SIS peptide mix than the wells of the remaining time points.

Interestingly, for all five lots of blood, the most pronounced changes in the determined protein concentrations occurred between the 0 h and 1 h time points, while there are only minor differences between 1 h up to 40 h of storage. A closer look into the hierarchical clusters reveals two small subsets of protein assays that seem to be affected by plasma processing delays: a first subset where a clear deviation from the time point 0 can be seen for all 5 blood lots and most time points and a second subset where the time points of only some of the blood lots show this deviation (Fig. 4).

Overall, the RSD results (>90% of peptides quantified had RSD values <30%) and heat map visualizations indicate that most proteins with quantifiable END concentrations were stable despite prolonged incubation of the whole blood samples at room temperature. Moreover, these results were

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**Fig. 2.** Distribution of RSD ranges for peptide concentrations quantified from five whole blood samples. RSDs were calculated for each peptide where a minimum of three out of ten peptide concentrations for time points 0 to 40 h were above the LLOQ. Peptides were considered to be stable when the RSD was <20%, semistable when the RSD was between 20% and 30%, and unstable/irreproducible when the RSD was >30%. A, RSDs for all peptide concentrations quantified from plasma thawed quickly. B, RSDs for all peptide concentrations quantified from plasma thawed overnight at 4 °C. C, RSDs calculated for peptide concentrations for O/N plasma samples, excluding concentration values for the 6 h time point of sample 4 and for the 1 h, 20 h, and 40 h time points of sample 5. LLOQ, lower limits of quantitation; O/N, overnight; RSDs, relative SDs.

**Fig. 3.** Peptide concentration percent differences for all time points compared to their 0 h time point. A–E, samples 1 to 5, plasma thawed quickly on ice. F–J, samples 1 to 5, plasma thawed overnight at 4 °C. An asterisk (*) indicates time points which showed unexpectedly low protein concentrations.
consistent between all five whole blood sample lots. Finally, thawing plasma either quickly on ice prior to immediate processing or O/N before processing appeared to result in comparable RSDs.

**Differences Between Quick-Thaw and O/N-Thaw**

The effect of thawing plasma O/N at 4 °C in the refrigerator prior to sample processing was assessed by comparing time point 0 h from the quick-thaw sample batch and time point 0 h from the O/N-thaw sample batch. The protein concentration values obtained from these samples showed excellent agreement for all five lots of whole blood assessed, with Passing-Bablok regression plots showing slopes ranging from 0.95 and 1.01 and Pearson’s r values of 1.00 (see Fig. 5).

Moreover, comparing the median %RSDs between ON and QUICK thaw conditions, calculated from at least three of five samples that had sufficient data points to calculate %RSDs (n = 159), showed good correlation, with a slope of 1.09 and an R² value of 0.90 (supplemental Fig. S2). This suggested that the higher variability of a small number of peptides is independent of the thawing condition.

**Stability of Surrogate Peptides to Quantify Plasma Proteins**

Of the 274 targeted peptides, 159 yielded sufficient quantitative data to determine a minimum of three RSD values across the five O/N thaw samples and three RSD values across the five quick thaw samples (Fig. 6 and supplemental Table S3). Of these 159 peptides, 139 (87.4%) showed median RSDs <20% and were thus considered stable. Only 14 of the 159 peptides (8.8%) had median RSDs of 20 to 30% and were therefore considered semistable. Finally, only 6/159 (3.8%) of the peptides showed median RSDs above 30% and were classified as unstable. The remaining 115/274 peptides had insufficient data points, that is, less than three quantifiable concentrations per peptide across the five sample lots, to assess stability. This result was expected because of the low concentrations of known disease biomarkers in plasma samples from healthy subjects (13) and was not attributable to rapid degradation, loss of signal due to prolonged storage at room temperature, or differences in sample processing. Additionally, the physicochemical peptide properties, such as presence of oxidizable residues in the peptide sequences, did not correlate with stability.
The surrogate peptides of proteins, such as plasma serine protease inhibitor, cystatin C, complement C5, phospholipid transfer protein, gelsolin, fibrinogen beta chain, and beta-2-microglobulin, showed RSDs of 18% or lower, and were therefore considered stable. In contrast, the surrogate peptides of proteins, such as lactotransferrin, transcription factor SOX 1, and platelet endothelial cell adhesion molecule, showed RSDs well above 30%, indicating that for these particular peptides, a delay in sample processing affects the determination of the original protein levels. A decrease in the measured surrogate peptide concentrations can be attributed to the general occurrence of protein degradation or modifications during prolonged processing times. While proteolysis can impact intact protein concentration measurements, based on our findings, peptide-based quantitation using validated MRM assays is substantially more robust toward protein degradation, as this will only affect the measured concentrations in cases where the target peptide itself is cleaved. Interestingly, however, some proteins such as S100-A2 and haptoglobin showed a clear increase in measured concentrations over time. Increases in measured protein concentrations after blood sample storage and processing delays have been documented. Vascular endothelial growth factor was measured by ELISA after delay at 4°C before centrifugation. Vascular endothelial growth factor was found to increase over time and mRNA quantified from peripheral blood mononuclear cell confirmed an increase in expression over time (30). Additionally, residual activities of

![Fig. 5. Comparison of thawing procedures through nonparametric regression analysis. Passing-Bablok regressions for the protein concentrations determined for five whole blood sample lots 1 to 5 (A–E), at room temperature for the 0 h time point. The regressions compare thawing quickly on ice versus overnight at 4°C. Insets show the data point for the serum albumin peptide LVNEVTEFAK.](image)

![Fig. 6. Overview of stable, semistable, and unstable peptide counts determined for quantifiable peptide concentrations across at least three of the five sample lots. “Insufficient data” indicates that protein assay stability was not assessable because of a lack of sufficient data points. Stable, semistable, and unstable categories were established based on median RSD ranges with <20%, 20 to 30%, and >30%, respectively. RSDs, relative SDs.](image)
endogenous enzymes might cause the removal or rearrangement of complex post-translational modifications (PTMs) causing further increases in measured protein concentrations (31). Indeed, there is evidence of residual enzymatic activity of enzymes such as β-glucuronidase and hexosaminidase, which are involved in sugar breakdown when blood collection is carried out in acid citrate dextrose tubes (32), as was the case in our study. This enzymatic activity can vary between individual blood samples but may be exacerbated by maintaining samples at room temperature for a prolonged period of time. Although the target proteotypic peptides were selected based on the absence of known PTMs, even changes in PTMs that are in close proximity to tryptic cleavage sites (such as the removal of phosphorylation sites (33) or the removal of glycans (34)) can lead to an increased digestion efficiency of the target peptide and thus result in an apparent increase in the measured protein concentration. We have previously demonstrated that optimum digestion conditions (particularly with regard to the duration of protein digestion), which lead to maximum peptide-recovery vary for individual plasma proteins and target peptides. Thus, any digestion protocol can only be a compromise, designed to yielding the best overall recovery for the target peptides (35).

CONCLUSION

Events such as the recent COVID-19 pandemic have highlighted the need to study protein stability in blood upon collection, since pressure on the health sector can result in delays in sample processing, potentially compromising sample integrity depending on downstream applications. Clinical samples represent, in many cases, a one-time sampling opportunity and therefore there is a need to determine the conditions under which samples can be trusted to provide accurate results for diagnostic and research purposes. In this study, we used a set of LC/MRM-MS assays for 270 human plasma proteins to investigate if storage of human whole blood at room temperature for up to 40 h would affect the measurement of protein concentrations by LC/MRM-MS.

Our results show that peptide-based protein quantitation by LC/MRM-MS is mostly unaffected by potential protein degradation related to suboptimal handling and storage of whole blood samples. The large majority (96%) of protein assays resulting in quantifiable END concentration in this study can be considered as stable (RSDs <20%) or semistable (20% < RSD <30%) across all time points, while only 4% of the protein assays were found to be affected by delays in plasma generation. Notably, for unstable assays, the most pronounced changes occurred between the 0 h and 1 h time points, while longer delays in plasma processing did not affect the measured peptide concentrations in a comparable way.

Based on our results, a stringent standard operating procedure for rapid sample processing might be crucial only for a small subset of the proteins targeted by our 270+ LC/ MRM assays. For the majority of the proteotypic peptides we used in our assay, however, prolonged storage of whole blood at room temperature appeared to have minimal effect on protein concentration values that were determined by LC/MRM-MS. In addition, whether plasma samples were thawed quickly on ice or O/N at 4 °C did not considerably affect the quantitative results, suggesting that either approach works for the targeted protein quantitation method used here. The LC/MRM-MS method used for this study relies on the quantitation of proteins based on surrogate peptide concentrations, which explains the expected successful concentration determination of most targets. Importantly, methods relying on antibody-based binding and recognition of intact, native proteins, such as ELISA, would likely fail to determine the original concentrations after prolonged storage at room temperature due to the denaturing and proteolytic activities characteristic of blood samples.

DATA AVAILABILITY

The MRM raw data are available via the public MS data repository PanoramaWeb (36), at https://panoramaweb.org/xv2YJx.url.

Supplemental data—This article contains supplemental data.

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Conflict of interest—C. H. B. is the CSO of MRM Proteomics, Inc. R. P. Z. is the CEO of MRM Proteomics, Inc. All other authors declare no competing interests.

Abbreviations—The abbreviations used are: ACN, acetonitrile; BSA, bovine serum albumin; CPTAC, Clinical Proteome Tumor Analysis Consortium; FA, formic acid; LC, liquid chromatography; LLOQ, lower limits of quantitation; MRM-MS, multiple reaction monitoring mass spectrometry; MS, mass spectrometry; NAT, natural, light; O/N, overnight; PTMs, post-translational modifications; QC, quality control; RSDs, relative SDs; SIS, stable isotope-labeled standard; SPE, solid-phase extraction.

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