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Robust, reproducible, and easy-to-implement plasma protein profiling workflow using high-flow UHPLC/MS/MS without depletion

Authors

David Sarracino, Scott Peterman
Thermo Fisher Scientific,
Cambridge, MA

Keywords

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Goal

To generate a robust and reproducible bottom-up protein profiling workflow that addresses throughput requirements for plasma biomarker discovery

Introduction

Plasma and serum are the two most highly utilized biofluids for diagnostic testing. As a working biological fluid, plasma functions to transport nutrients, hormones, and cells. It also plays an active role in innate immune response and clotting. The function and composition of plasma are vital to health, and plasma is a major carrier for biomarkers of disease. Plasma consists of salts, drugs, metabolites, and proteins whose concentrations span 12 orders of magnitude. In a study of protein-based biomarkers, 20,000 proteins are reported in plasma. However, the top 20 proteins constitute 98% of the mass of all proteins in plasma, rendering access to identifying and quantifying low concentration proteins of interest difficult as a routine practice. Depletion columns and extensive protein and peptide-based fractionation significantly increase the accessible depth to low concentration proteins, but at price of increased cost, additional labor, and decreased analytical reproducibility.[1]

To facilitate the identification and quantification of plasma biomarkers for clinical research, we developed a highly robust liquid chromatography mass spectrometry-based protein profiling workflow for the analysis of undepleted plasma. This method specifically addresses several key issues with analyzing undepleted plasma to ensure the proper balance between the robustness of the analytical platform and the depth of protein coverage. In this technical note, we focus on the durability of the chromatography system to ensure its capability of handling a large volume injection of plasma samples, hundreds of injections before deep cleaning, and the minimum of carryover.

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Experimental

Improving UHPLC performance

In all UHPLC systems, there are recurring service calls for failed check valves due to the exposure of pure acetonitrile.[2] Modern check valves made of ceramic composites reduce the frequency of sticky check valves, but pure acetonitrile at routine 800 bar pressure can still polymerize on check valve surfaces, causing them to stick. Periodically flushing the system with a water/isopropanol solvent blend can eliminate this issue. However, for systems run continuously, this downtime is costly. Another alternative solution is to add a certain amount of water to acetonitrile mobile phase to reduce check valve fouling, but this also reduces the eluotropic potential of the organic phase leading to column failure due to insufficient washing. To overcome this challenge, we implement a solvent blend with 10% water, 10% isopropanol, and 80% acetonitrile to allow for continuous use of the UHPLC without the need for periodic regeneration of the check valve surfaces. The addition of 2% methanol to the aqueous mobile phase A improves the pumping accuracy by reducing the surface tension in the pump head and check valves.

Optimizing liquid chromatographic performance for high flow capacity

In this workflow, following LC setup was used for high flow capacity plasma protein identification:

1. Thermo Scientific™ Vanquish™ Horizon UHPLC system
2. Trap column with 100 Å pore size, 3 µm particle size, and 2.1 × 50 mm column length
3. Thermo Scientific™ Acclaim™ C18 120 column (two, connected in series)

Trap column

Pairing chemical properties of the trap and analytical columns is critical for high peak capacity applications. A correct pairing increases the separation capacity from the trap column and the refocusing capability of the analytical column after peptides are transferred from the trap column to the analytical column. Therefore, matching trap and analytical column chemistries can greatly amplify the “at load peak capacity” of the LC system. Utilization of the trapping column chemistry in which the k’ (capacity factor for a compound on a phase) for peptides is much lower than that of the k’ for the same peptides on the analytical columns facilitates the refocusing of peptides on the analytical column and prevents the analytical columns from being overloaded. It also allows high abundant peptides to be eluted onto the analytical column gradually over the entire gradient. To achieve better transition between the trapping and analytical columns and to improve the trapping chemistry kinetics, a positive temperature offset on the trap column using the Vanquish Horizon UHPLC system was used. Vanquish UHPLC is the only high performing liquid chromatography system with an active solvent preheating system. The workflow detailed here drastically reduces sample loading times by increasing the sample loading flow rate to 1 mL/min at 80 °C with high efficiency while experiencing only a minimal increase in backpressure.

The polystyrene-divinylbenzene phase with 3 µm particle size and 100 Å pore trap column is capable of retaining its functionalities and performance parameters even in the presence of aggressive cleaning solvents. The columns also show high stability in high temperatures and were less prone to clogging while withstanding high velocities during sample loading.

Analytical column

The Acclaim C18 column is an 18% carbon load phase with an excellent peak capacity and refocusing capability. Two 2.1 mm × 250 mm columns are connected in series to form a 500 mm analytical column with a back pressure of about 750 bar for all three columns (trap column and analytical columns) in series.

Sample loading and gradient

Digested plasma (90 µg) was loaded on the trap column with a flow rate of 1 mL/min, and a 52 min gradient was used for peptide separation at 250 µL/min. The Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (PRTC, cat# 88320) was spiked into every sample for retention time monitoring and signal normalization.

An example of QC and plasma sample running sequences is shown in Table 1.

Minimizing the sample carryover

After a single injection, an HPLC column (and all the tubing before it) ceases to be a C18 column and becomes a mixture of all the components of the sample including lipids, DNA, large proteins, undigested protein aggregates, and structural proteins. This leads to increased system backpressure, shifting retention times, increasing carryover (further), and overall decreasing column performance over time. Since the trap column is loaded at high flow rate, the pore restriction on the trap column acts as an MWCO filter, proportional to the loading flow rate. The hydrophobic components that contribute to carryover can be eliminated by stopping the gradient before hydrophobic components elute to the analytical column. Lastly, it's important to inject a cleaning blank sample that cleans the autosampler, tubing, and trap column, while flushing the analytical column separately.

Table 1. QC and plasma sample running sequences

Run Type	Run Method	Injection Volume/Solvent
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
PRTC QC 1	15 min	5 µL 100 fm/µL PRTC in 2% methanol and 0.1% formic acid
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
System Suitability Test	52 min + 8 min clean	90 µg digested pooled pllasma standard + PRTC
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
Pooled QC Control	52 min + 8 min clean	90 µg digested plasma pooled from study + PRTC
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
PRTC QC 2	15 min	5 µL 100 fm/µl PRTC in 2% methanol and 0.1% formic acid
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
Sample 1 (replicate 1)	52 min + 8 min clean	90 µg digested plasma sample + PRTC
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
Sample 1 (replicate 2)	52 min + 8 min clean	90 µg digested plasma sample + PRTC
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial
PRTC QC 3	15 min	5 µL 100 fm/µL PRTC in 2% methanol and 0.1% formic acid
Equilibration	Equilibration	90 µL formic acid from 10 mL reagent vial

In this workflow, two injections are carried out per run, splitting the gradient into an analytical separation portion, and cleanup/equilibration portion (Figure 1). The cleanup/equilibration portion of the run is to inject 45 µL of formic acid only to the trap column. The intercolumn divert valve is switched back after the trap column is clean, and then all columns are washed with high organic mobile phase and equilibrated back to initial conditions with aqueous mobile phase. Highly hydrophobic compounds never get eluted onto the analytical column. This cleanup procedure ensures that minimal carryover is observed in large scale sample runs (>100 runs).

Results and discussion

The high-flow LC setup yields highly reproducible retention time profile

Using the high-flow LC setup, 100 fmol of the PRTC mix was spiked in digested undepleted plasma to determine retention time (RT) variations (%CV) of PRTC peptides in a large number repeated injections. Even in a shallow gradient of 8–32% over 50 min, the outstanding reproducibility and robustness was observed using this high-flow LC setup, with <4% CV of RT across 130 repeated injections (Figure 2).

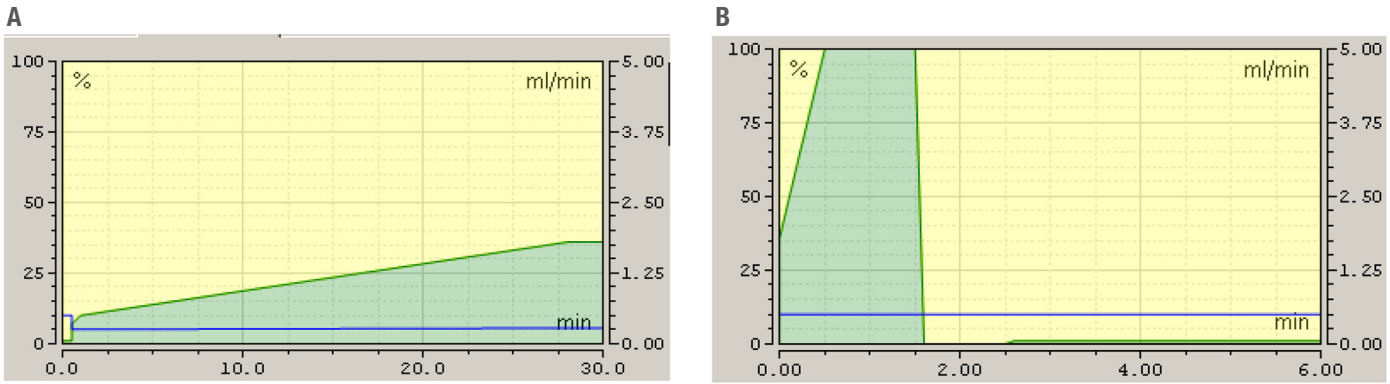

Figure 1. Decoupling the A) analytical LC program and B) regeneration LC program with two injections per sample

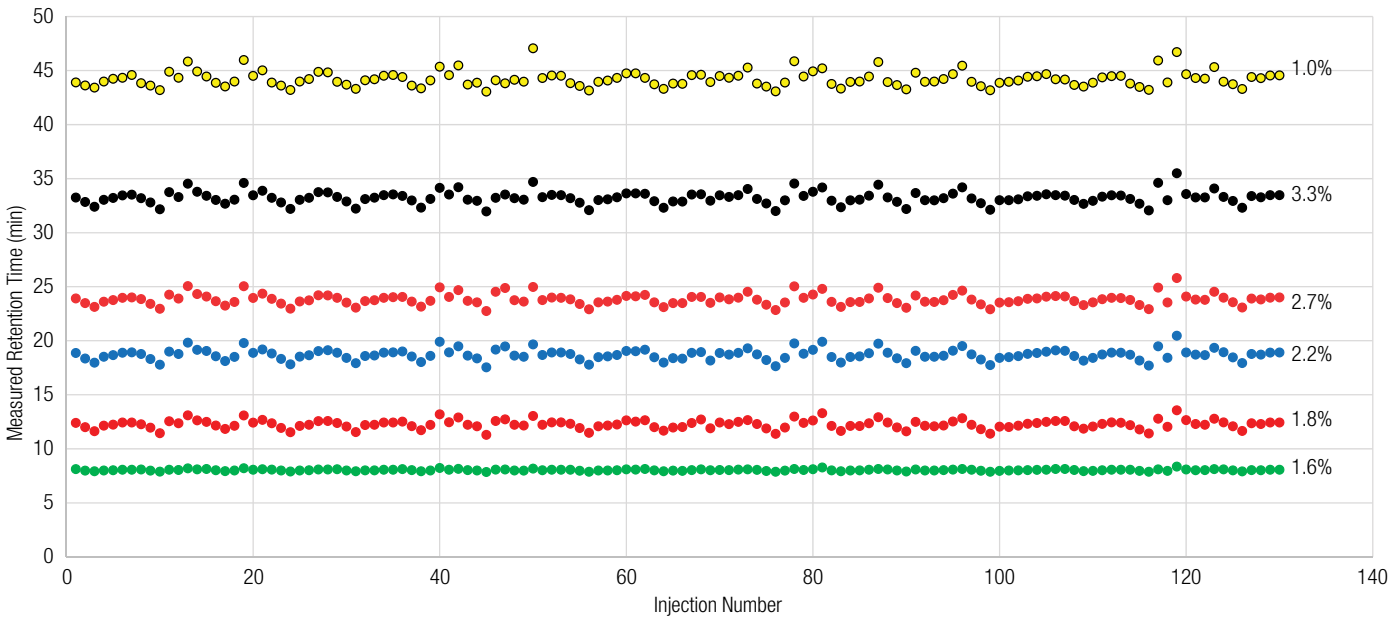

Figure 2. PRTC peptide retention time reproducibility across 120 injections in depleted plasma

To further establish the repeatability, reliability, and reproducibility of this workflow, the next set of experiments focused on the repeatability of plasma proteome profiling using the high-flow LC setup to analyze 16 individual blood samples collected from the same donor. Three aliquots of plasma were processed from each blood draw as technical replicates for protein assignments. On average, 460 proteins and 2222 peptides were quantified per tube, with an additional 136 proteins and 513 peptides identified per tube (Figure 3).

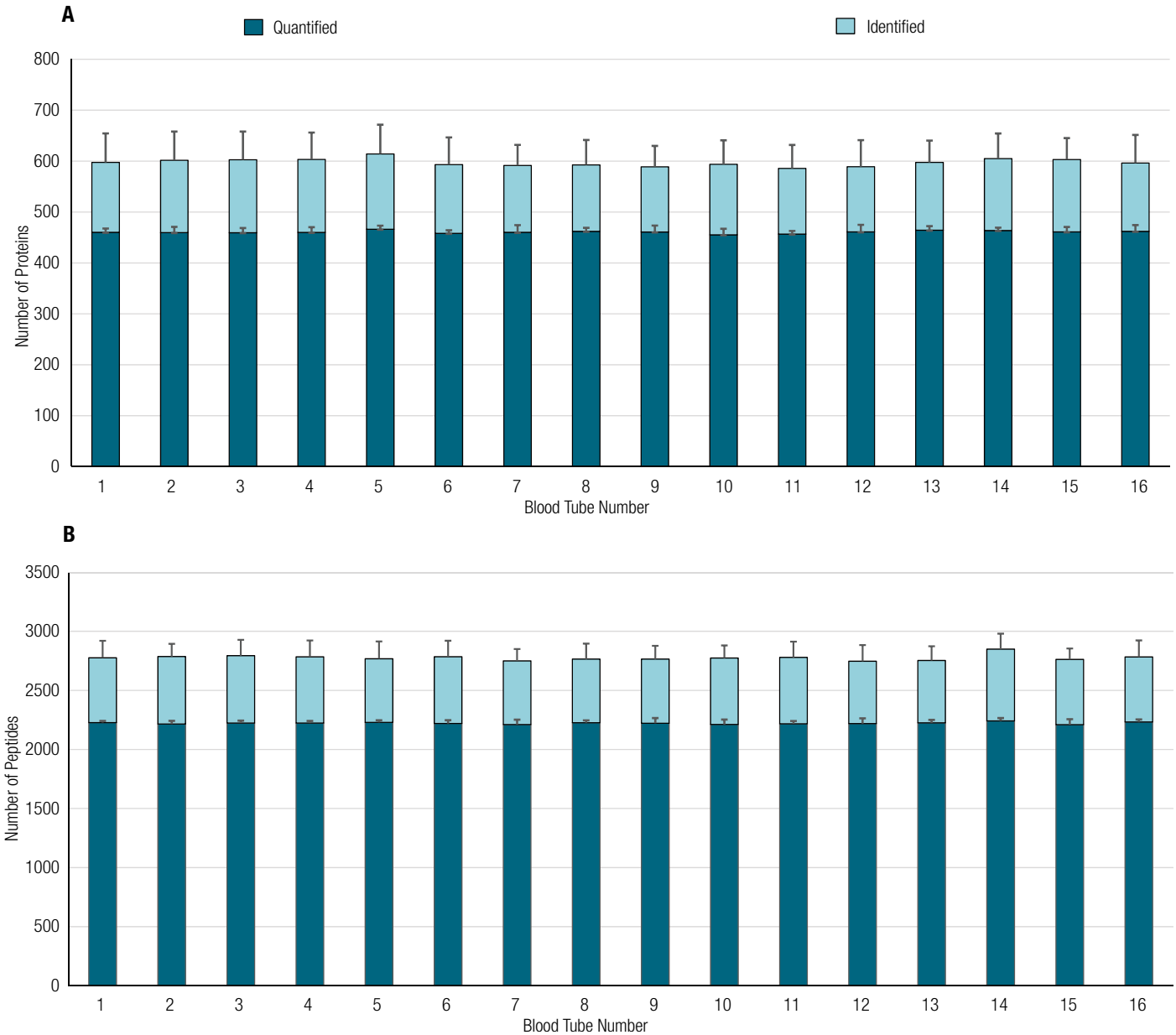

Figure 3. Reproducibility of protein (A) and peptide (B) quantification and identification across replicate blood draws

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Conclusion

The LC method described in this workflow was coupled with a high-resolution mass spectrometer such as the Thermo Scientific™ Q Exactive™ series mass spectrometer to generate robust and reproducible data from a large number of undepleted plasma samples. The method includes high-performance HPLC pumps, customized solvent blends, aggressive cleaning cycles, and paired column chemistry to overcome several challenges typically faced in the analysis of undepleted plasma. This robust and repeatable high-flow plasma profiling workflow can enable researchers to reproducibly quantify a large amount of plasma proteins while reducing the effect of sample size due to technical variabilities. The typical workflows involved in protein and peptide identifications can be further improved with the application of advanced data acquisition enabled by search methods such as spectral library matching.

References

1. Anderson, N.L. et al. *Mol Cell Proteomics* **2002,** *1*(11), 845–67.
2. Dolan, J.W. et al. *LCGC Europe* **2006,** *19*(3), 140–144.

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