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Application of volumetric absorptive microsampling for robust, high-throughput mass spectrometric quantification of circulating protein biomarkers

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ABSTRACT

Volumetric absorptive micro sampling (VAMS™) allows accurate sampling of 10 μL of blood from a minimally invasive finger prick and could enable remote personalized health monitoring. Moreover, VAMS overcomes effects from hematocrit and sample heterogeneity associated with dried blood spots (DBS). We describe the first application of VAMS with the Mitra® microsampling device for the quantification of protein biomarkers using an automated, high-throughput sample preparation method coupled with mass spectrometric (MS) detection.

The analytical performance of the developed workflow was evaluated for 10 peptides from six clinically relevant proteins: apolipoproteins A-I, B, C-I, C-III, E, and human serum albumin (HSA). Extraction recovery from blood with three different levels of hematocrit varied between 100% and 111% for all proteins. Within-day and total assay reproducibility (i.e., 5 replicates on 5 days) ranged between 3.2–10.4% and 3.4–12.6%, respectively. In addition, after 22 weeks of storage of the Mitra microsampling devices at −80 °C, all peptide responses were within ± 15% deviation from the initial response. Application to data-independent acquisition (DIA) MS further demonstrated the potential for broad applicability and the general robustness of the automated workflow by reproducible detection of 1661 peptides from 423 proteins (average 15.7%CV (n = 3) in peptide abundance), correlating to peptide abundances in corresponding plasma (R = 0.8383).

In conclusion, we have developed an automated workflow for efficient extraction, digestion, and MS analysis of a variety of proteins in a fixed small volume of dried blood (i.e., 10 μL). This robust and high-throughput workflow will create manifold opportunities for the application of remote, personalized disease biomarker monitoring.

1. Introduction

Dried blood can be collected in small amounts from a minimally invasive finger prick, which, in contrast to serum or plasma specimens, eliminates the need for trained phlebotomists, hospital visits, elaborate sample processing, and controlled transportation. The collection of dried blood has the potential to enable sample collection in remote locations, which would increase patient participation and sample access. Dried blood collection, primarily performed using dried blood spots (DBS), involves the placement of small volumes of capillary blood onto special paper cards, and is particularly well-established for the analysis of small molecule biomarkers, e.g., in newborn screening [3]. However, DBS suffer from sample heterogeneity and hematocrit issues that cause non-homogeneous spreading of blood and analyte onto the filter paper and complicate analysis of a standardized volume [4]. Volumetric absorptive microsampling (VAMS™) offers accurate sampling of a fixed small volume of blood (e.g., 10 μL), while overcoming the negative effects associated with DBS [5,6]. Numerous studies have successfully applied VAMS with the Mitra® microsampling device to the bioanalysis of small molecules and have reported better accuracy and correlation to venous blood than that found with DBS [7–10].

Remote blood sampling to quantitate protein biomarkers is,

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however, less well-established. Proteins are major drivers of many disease pathways, and remote or longitudinal sample collection would offer great opportunities in population-based or personalized health monitoring [1,2]. In recent years, targeted quantification of protein biomarkers in DBS has been increasingly explored by taking advantage of the specificity and multiplexing capacity of mass spectrometry (MS) [11–17]. The application of VAMS with the Mitra® microsampling device for quantification of protein biomarkers has, nonetheless, not yet been reported.

MS-based analysis of protein biomarkers typically involves digestion of the protein by a proteolytic enzyme and detection of representative peptides by: (**i**) selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) for targeted quantification, (**ii**) data-dependent acquisition (DDA) for protein identification, or (**iii**) data-independent acquisition (DIA) for protein identification and SRM-like quantification. SRM has been introduced into the clinical laboratory as a more specific alternative for antibody-based clinical protein assays [18], additionally enabling the quantification of specific protein variants [19,20] or the quantification of multiplexed biomarker panels [21,22]. Important considerations in the clinical application of SRM-based laboratory-developed tests for protein biomarkers include: (**i**) reduction of the sources of analytical errors [23,24], (**ii**) automation or on-line coupling of sample preparation [21,25–27], and (**iii**) metrological traceability of measured protein concentrations [28]. In line with the improvements in robustness, precision, and trueness of clinical MS for protein quantification, DIA is rapidly evolving to provide precise, unbiased (i.e., without *a priori* selection) quantification of large numbers of proteins by *targeted* extraction of continuously acquired MS/MS spectra [32,33]. Whereas DDA provides high resolution MS/MS spectra and, hence, high specificity of identification for any compound that exceeds a detection threshold, DIA provides SRM-like MS/MS peak profiles that can be (also retrospectively) integrated for *more-precise* quantification of, theoretically, *any compound* as long as the compound is defined by its specific mass transitions and stored in a spectral library [34].

DBS are reported to have been applied to SRM [11–17] and DDA [35], but not to global quantification by DIA. In this manuscript, we describe the development of an automated platform for efficient extraction and digestion of proteins from the Mitra microsampling device with applicability to both SRM and DIA MS. The combination of accurate volumetric sampling and automated sample preparation follow the current trends to: (**i**) improve the accuracy of targeted and discovery-based MS [23,24], and (**ii**) explore the potential of remote blood collection for longitudinal monitoring of multiplexed biomarker panels [1].

The developed analytical pipeline offers opportunities to integrate accurate remote fingerprick blood collection with the Mitra microsampling device into population-scale biomarker discovery studies, as well as longitudinal clinical risk assessment based on targeted protein panels. The analytical performance of the developed workflow has been carefully evaluated for 10 peptides from six clinically relevant proteins, including the cardiovascular risk-associated apolipoproteins (i.e., apoA-I, apoB, apoC-I, apoC-III, and apoE), as well as human serum albumin (HSA), using an SRM assay that includes stable-isotope labeled peptide analogs for each targeted peptide and exogenous beta-galactosidase (BGAL) for process quality control. To evaluate the broader applicability and global robustness of the workflow, global protein quantification of dried blood was performed using a DIA assay optimized for plasma biomarker discovery.

2. Experimental section

2.1. Loading of Mitra tips

Six pools of human whole blood (with K_2EDTA preservative) were obtained from Bioreclamation I VT (Chestertown, MD, US) over a period of 11 months. All blood pools were stored at 4 °C and used within seven days of collection. Mitra® microsampling devices (Neoteryx, Torrance, CA, US) with 10 µL volumes were loaded with blood by dipping the tips into a 1.5 mL Eppendorf tube filled with blood. Care was taken that the Mitra tips only touched the liquid surface and were not submerged. The tips were held at the surface until fully colored red, and then for an additional 3 s to ensure a complete fill. The filled Mitra tips were allowed to dry for at least 16 h at room temperature and stored until further use in a closed container at room temperature, unless otherwise specified.

2.2. Preparation of combined heavy peptide working solution

For each targeted peptide, a stable-isotope labeled (heavy) peptide analog was included. The 13 stable-isotope labeled peptides, with $^{13}C_6{}^{15}N_4$ labeled arginine or $^{13}C_6{}^{15}N_2$ labeled lysine as C-terminal amino acid residues (New England Peptides, Gardner, MA, US), were obtained as lyophilized powder and reconstituted in 20% (v/v) acetonitrile, 0.1% (v/v) formic acid in water. The individual heavy peptide solutions were combined to make a heavy peptide working solution that provided, as based on amino acid analysis, 1250 pmol DDNPNLPR and LVNEVTEFAK (HSA), 100 pmol WVGYGQDSR, IDPNAWVER, and GDFQFNISR (BGAL), 50 pmol THLAPYSDELR (apoA-I), 10 pmol FPEVDVLTK and GFEPTLEALFGK (apoB), 10 pmol TPDVSSALDK (apoC-I), 10 pmol GWVTDGFSSLK (apoC-III), and 5 pmol SELEEQLTPVAEETR, LGPLVEQGR, and AATVGSLAGQPLQER (apoE) per sample. The combined heavy peptide working solution was aliquoted into single-use portions and stored at
−80 °C.

2.3. Preparation of reagents for automated extraction and digestion

20% (w/v) octyl-beta-glucopyranoside (OGS, Sigma-Aldrich) in water, 50 mMol/L Tris (2-carboxyethyl) phosphine (TCEP, Thermo Scientific, Waltham, MA, US) in water, 200 mMol/L methyl methane thiosulfate (MMTS, Thermo Scientific) in isopropanol, and 5 mg/mL BGAL from E. Coli (Sigma-Aldrich) were prepared as single-use aliquots and stored at -80 °C. A stock solution of 10% (v/v) formic acid was prepared by dilution of a 1 mL ampule of formic acid (Optima LC-MS, Fisher Scientific) in 9 mL water. The stock solution was stored in the dark at room temperature. On each day of analysis, the above-described reagents for denaturation, reduction, and alkylation were thawed and placed on-deck on the robotic liquid handling platform (Biomek NX^P Span-8 Workstation, Beckman Coulter Life Sciences, Indianapolis, IN, US). In addition, the buffer for extraction and digestion was freshly prepared by dissolving Tris pre-set crystals (Sigma-Aldrich), in 4 mMol/L $CaCl_2$ (Sigma-Aldrich) to make a 100 mMol/L Tris, 4 mmol/L $CaCl_2$ buffer with pH 8.5. The combined heavy peptide working solution was diluted with buffer resulting in 16.1 µL of the combined heavy peptide working solution per 127.5 µL buffer. Lastly, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sciex, Framingham, MA, US) was dissolved in 0.1% (v/v) formic acid at a concentration of 2 µg/µL and stored on-deck until use.

2.4. Automated extraction and digestion workflow

The automated protocol for extraction and digestion was adopted from a previously optimized workflow for trypsin digestion of plasma on the same Biomek NX^P Workstation[25]. First, a 150 µL mixture composed of 15 µL 20% w/v OGS, 25 µL 50 mmol/L TCEP, and 110 µL 100 mmol/L Tris, 4 mmol/L $CaCl_2$ buffer pH 8.5 was added to each well of a 1 mL deep well titer plate (Beckman Coulter), hereafter named as *sample plate*. Mitra tips were removed manually from the Mitra microsampler body by gently pushing with the long side of a needle while holding the tip to the edge of the destination well. The sample plate was then placed on the shaking peltier ALP (Inheco, Martinsried, Germany) for a one hour incubation with rigorous shaking (i.e., 1200 RPM) at