thermoscientific

Analysis of Hemoglobin Variants by PaperSpray Mass Spectrometry for Clinical Research

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ABSTRACT

Purpose: The potential for using PaperSpray mass spectrometry (PSMS) for the analysis of Hb variants in clinical research is tested. PSMS is attractive for hemoglobin variant analysis because the analyte is at high concentration in blood, the test is qualitative in nature and eliminating sample preparation steps is of benefit to speed the sample to knowledge workflow.

Methods: A Prosolia Velox360™ PaperSpray® system was coupled to Thermo Scientific™ high-resolution accurate mass (HRAM) mass spectrometers for the identification of specific peptides indicative of different hemoglobin proteins. A bottom-up enzymatic trypsin digestion method and a top-down intact protein method were tested. The tryptic digestion was performed directly on the Prosolia Velox paper cartridge in a short amount of time.

Results: The potential for using PaperSpray mass spectrometry (PSMS) for the analysis of Hb variants in clinical research is demonstrated with a sickle cell variant standard (HbS) spiked in blood. Three peptides indicative of either variant or normal hemoglobin were identified by PSMS and MS/MS in a HRAM instrument. The intact protein was also detected by PSMS.

INTRODUCTION

PaperSpray mass spectrometry (PSMS) is a technique that works from a physiological fluid sample deposited on paper (such as a dried blood spot). Electrospray mass spectrometry is achieved after rewetting the sample and adding enough solvent for a 1 min experiment. PSMS is attractive for hemoglobin (Hb) variant analysis because the analyte is at high concentration in blood, the test is qualitative in nature and eliminating sample preparation steps is of benefit as it speeds the sample to knowledge workflow. Removing liquid chromatography (LC, separation step) from the conventional workflow of LCMS provides structurally informative mass spectra in a significantly shorter period.

Hemoglobin analysis is relevant to newborn screening, prenatal screening and transfusion monitoring (1). Most popular techniques used for Hb measurements are electrophoresis, HPLC, molecular genetics, LC-MS/MS bottom-up and LC-HRAM-MS top down approaches.

In this work, the potential for using PSMS for the analysis of Hb variants in clinical research is demonstrated with a sickle cell variant standard (HbS) spiked in blood.

MATERIALS AND METHODS

Sample Preparation

The mass spectrometry method was developed with a hemoglobin S standard (dissolved to 180 mg/mL in aqueous TRIS buffer pH 7.5). Trypsin was dissolved to a final concentration of 5 mg/mL by adding 20 µL 50:50 MeOH/H_2O to 100 µg . Seven µL of the HbS standard were spotted on the paper cartridge followed by 4.5 µL of dissolved trypsin protease added on top. Human whole blood was spotted on the paper cartridge (12 µL) and allowed to dry in an oven at 37 degrees Celsius for 20 min prior to enzymatic digestion. Normal blood was spiked with the hemoglobin S variant standard and allowed to dry under the same conditions. 4.5 µL of dissolved trypsin protease were added to each dry blood spots. Trypsin digestion was allowed to process for 10 min at room temperature, adding small volumes of water to keep the mix wet (Fig. 1).

Pierce™ trypsin protease, MS grade (100 µg), was purchased from Thermo Fisher Scientific. Lyophilized human hemoglobin S, ferrous stabilized standard (>90% ferrous Hb when dissolved) was purchased from Sigma-Aldrich (St. Louis, MO). K_2 EDTA-treated human blood was purchased from Bioreclamation IVT (Baltimore, MD).

The HbS protein standard for the nanoLC mass spectrometry experiment was digested using the Thermo Scientific™ SMART Digest™ Kit.

Test Methods

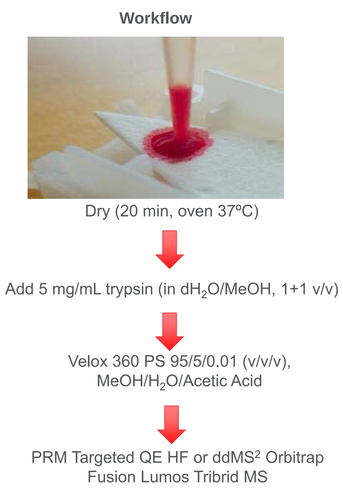
A Thermo Scientific™ EASY-nLC™ 1000 HPLC system and Thermo Scientific™ EASY-Spray™ source with Thermo Scientific™ EASY-Spray™ LC column (PepMap RSLC C18 2um, 15 cm x 50 µm i.d.) was used to separate peptides with a 5-40% acetonitrile gradient in 0.1% formic acid over 30 min at a flow rate of 200 nL/min. The sample was analyzed using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer with Data Dependent™ MS^2.

A Prosolia Velox™ 360 PaperSpray® system was coupled to a Thermo Scientific™ Orbitrap™ Q Exactive™ HF mass spectrometer. A targeted experimental method with an inclusion list (parallel reaction monitoring, PRM) was used for the bottom-up study. One minute PaperSpray experiments were conducted where different mass spectrometry experiments were performed (e.g., SIM, high energy collisional dissociation (HCD)). The Prosolia Velox 360 source was then coupled to the Orbitrap Fusion Lumos Tribrid mass spectrometer for direct analysis of undigested hemoglobin from the Velox cartridge. PaperSpray extraction solvent was 95/5/0.01 methanol/H2O/acetic acid. For intact protein analysis, 5% 100 mM ammonium acetate comprised the aqueous portion of the spray solvent.

Data Analysis

Thermo Scientific™ Xcalibur™ Qual Browser and Skyline (2), a free, open source software, were used for data analysis.

Figure 1. Sample preparation workflow. Whole blood was spotted directly on the Velox cartridge and allowed to dry for 15 min at 37C.



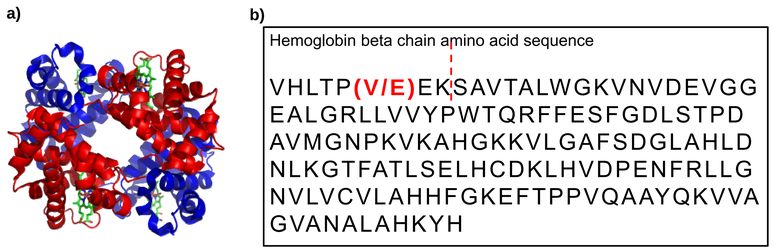
RESULTS

Human hemoglobin protein structure

The protein structure of human hemoglobin is depicted in Figure 2a. Human hemoglobin is an iron-containing metalloprotein whose function is to distribute oxygen in red blood cells in the body. It is composed of α and β subunits (red and blue, respectively in the figure) and the iron-containing heme groups (green) folded into a tertiary structure.

The amino acid sequence of the beta chain subunit is shown in Figure 2b. The difference between normal and sickle cell hemoglobin is one amino acid: glutamic acid (E) for normal and valine (V) in sickle cell (Fig. 2), in the beta chain. Enzymatic cleavage of proteins by trypsin occurs after lysines (K) and arginines (R). Therefore the difference between normal Hb and the HbS variant will occur within the first tryptic peptide.

Figure 2. a) Human hemoglobin protein structure: α and β subunits are in red and blue, respectively, and the iron-containing heme groups, in green. b) Amino acid sequence of hemoglobin beta chain. The amino acid difference between HbS (sickle cell) and normal hemoglobin HbA is one amino acid, shown in red. First tryptic peptide indicated by broken red line.



Previous work by PaperSpray mass spectrometry

Proof of concept work was previously presented at MSACL US 2017 (2). In that work, direct tryptic digestions of normal vs. variant Hb were allowed to react in a Velox PaperSpray cartridge for 10 min at ambient temperature. Analysis was made by PSMS by coupling a Velox 360 PaperSpray ion source to a Thermo Scientific™ Q Exactive™ Focus mass spectrometer. Two peptide fragments were found to distinguish HbS (variant Hb) from normal HbA0 using MS^2 fragmentation.

In the present work we aimed to identify at least three characteristic fragments to better identify peptide 1 following tryptic digestion. The first experiment was by nano LC MS on an Orbitrap Fusion Lumos Tribrid mass spectrometer to identify all potential MS^2 fragments (Figs. 3 and 4).

Three of the peptides characteristic of HbS were identified by PSMS using targeted MS^2 experiments in a high resolution accurate mass instrument (Fig. 5). Figure 6 shows selected ion monitoring (SIM) spectra for peptide 1 in blank blood and blood spiked with HbS.

The intact protein for the sickle cell variant was also identified via a top down approach and full MS spectra (Fig. 8). Both the alpha and beta chains are clearly detected.

Figure 3. Theoretical MS/MS fragments for tryptic peptide 1 of: a) normal beta chain in HbA0 (VHLTPEEK) and b) beta chain in HbS (VHLTPVEK). In red are the fragments that were observed in this study and reference 2.

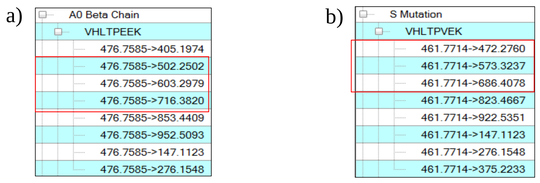


Figure 4. Analysis of hemoglobin variant HbS by nano LCMS and data dependent in the Orbitrap Fusion Lumos Tribrid MS. The resulting spectrum shows three signature fragments of HbS by MS^2. Characteristic fragments from this experiment are then transferred to a targeted PRM method by PSMS for analysis in a Q Exactive MS.

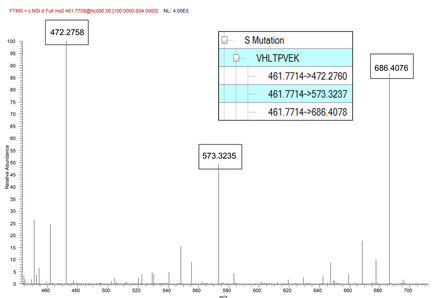
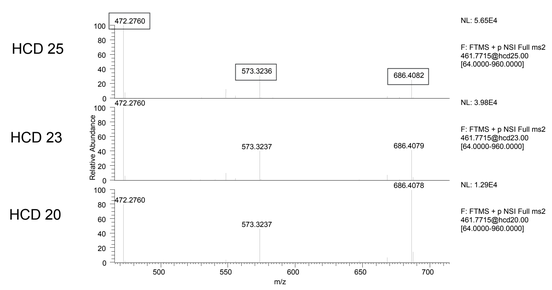


Figure 5. Hemoglobin HbS standard digested directly on paper and analyzed by PaperSpray MS. The same three signature fragments from the nanoLC experiment were detected. Q Exactive HF MS^2 spectra optimizing for high collision dissociation (HCD) energy.



RESULTS (continued)

Figure 6. SIM spectra for peptide 1 in blank blood and blood spiked with HbS. Peptide VHLTPEEK characteristic of normal Hb appears in both the blank blood and the mix. Peptide VHLTPVEK characteristic of HbS appears only in the mix.

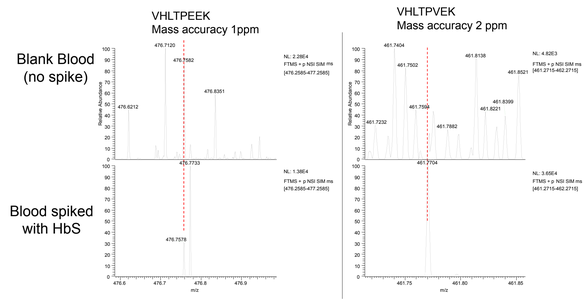


Figure 7. HbS standard spiked in whole blood and spotted on paper cartridge. Tryptic digest on dry blood spot, analysis by PaperSpray MS on Q Exactive HF mass spectrometer. MS^2 spectrum shows characteristic fragments for HbS.

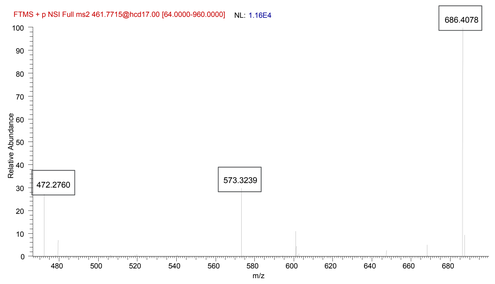
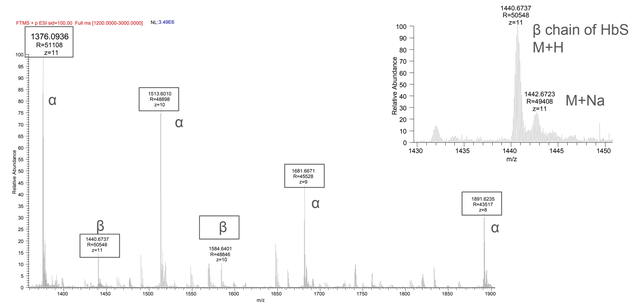


Figure 8. Intact HbS protein standard by top-down approach at 120,000 resolving power (FWHM @ *m/z* 200) and PaperSpray coupled to an Orbitrap Fusion Lumos Tribrid MS.



CONCLUSIONS

- Previous, proof of concept work has been successfully reproduced and expanded on.
- Tryptic digestion of dry blood spots processed in the Velox 360 paper cartridge and analysed directly from paper has produced mass spectrometry-detectable peptides characteristic of either normal haemoglobin or a variant spiked in blood.
- Use of targeted MS^2 in conjunction with high resolution accurate mass adds selectivity and provides useful data without chromatographic separation.
- Analysis of intact hemoglobin protein by a top down approach using PaperSpray mass spectrometry was also shown with the HbS standard sample (Fig. 8).
- This work opens-up PaperSpray technology for further investigation with peptides and proteins.
- Future work will develop a method for the detection of glycated hemoglobin, the HbA_{1C} variant.
- It is also of interest to develop these methods in a triple quadrupole mass spectrometer.

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REFERENCES

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2. MSACL US 2017. Corporate Workshop, Thermo Fisher Scientific. Paperspray MS in clinical research: early experiences in a U.K. clinical laboratory, *Lewis Couchman, Viapath Analytics, Kings College Hospital, London, UK*

TRADEMARKS/LICENSING

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