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Semi quantitative detection of signature peptides in body fluids by liquid chromatography tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

This study covers a modified semi-quantitative approach to detecting signature peptides for body fluid identification. A liquid chromatography tandem mass spectrometer normally used for toxicology was adapted to detect target ion transitions for five semen or saliva specific peptides. Peptide concentrations were measured based on a mixture of synthetic peptide standards. Samples were processed using a three-hour trypsin digestion and Microcon membrane filtration. This method generates PCR compatible DNA and peptide fractions that can be typed without any further treatment. Preliminary validation tests covered stains on different substrates, semen/saliva mixtures, limit of detection, and repeatability. All signature peptides were present at different concentrations, varied amongst donors, and were tissue specific. Saliva peptides were detected at lower concentrations and had a higher limit of detection (LOD). Semen peptides had higher concentrations and were detected even as a minor component in a mixture. All semen peptides and all, but one, saliva peptides were detected on the various substrates. Semen peptide concentrations had relative standard deviations (RSDs) lower than 20%, indicating high repeatability, different from saliva where higher RSDs were observed. DNA fractions did not show signs of degradation or PCR inhibition.

1. Introduction

Advances in mass spectrometry instrumentation have made universal peptide-based assays for body fluid identification more convenient and feasible. However, published peptide-based body fluid assays employ instrumentation that utilizes high resolution mass spectrometry (*e.g.* matrix assisted laser desorption ionization - time of flight), which are expensive and may be difficult to obtain for most crime labs [1–3]. Adapting low resolution mass spectrometry for peptide-based analysis may be an alternative since many crime laboratories already utilize this technique for toxicological analyses. In this study, we developed a semi-quantitative method to detect multiple signature peptides in saliva and semen in a multiplex format. Optimal conditions for the Shimadzu LCMS-8050 with a triple quadrupole mass spectrometer (QqQ) were established by employing peptide reference standards. Biomarkers were adopted [3] and combined in a multiplex as follows: for saliva, one signature peptide for histatin 1 (HIST) and two signature peptides for submaxillary gland androgen regulated protein 3B (SMR3B); and for semen, two signature peptides for semenogelin 1 (SEMG1). Synthetic peptides of the five biomarkers were used to determine at least 2 targeted product ions in multiple reaction monitoring (MRM) mode. To make the assay less time consuming and simultaneously recover the DNA component in a body fluid stain, the standard overnight trypsin digestion was reduced to a three-hour incubation and purification was based on a Microcon DNA FastFlow filtration step. The latter has a 100 kD cut off, which means DNA is retained by the membrane while peptides are in the flow through [4,5]. The resulting assay was tested on liquid samples for repeatability and sensitivity, and saliva and semen stains for a mixture study and substrate effects.

2. Materials and methods

Semen was purchased from a commercial provider (Lee Biosolutions) and saliva was collected from volunteers after CUNY IRB approval. Five microliter of semen and saliva were spotted on cotton (Puritan), polyester (Fisherbrand), and CEP (Gentuery) swabs. White cotton t-shirt, black cotton t-shirt, blue denim, tissue paper, and polyester t-shirt containing 5 µL of semen or saliva stains made in 2016 were also examined. Half of 10 µL semen stains on cotton swabs and white T-shirt spotted in 2014 were tested as well. Liquid semen and saliva were used to create mixed stains (5:1, 2:1, 1:1, 1:2, 5:1) on white cotton and cotton swabs, and aliquoted for the dilution series and repeatability tests.

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Liquid samples, stains, and swab cuttings were incubated in 100 µl of digestion buffer 50 mM NH_4HCO_3, 0.01% ProteaseMAX (Promega), 5 mM dithiothreitol (DTT, Promega) at 56 °C for 20 min on a shaker at 1400 rpm. Next steps were addition of 3 µL of 0.5 M iodoacetamide (IAA, Sigma), 20 min room temperature (RT) incubation in the dark, addition of 1 u L of 0.5 M DTT, and 20 min RT incubation in the dark. This was followed by adding 0.1 µg Sequencing Grade Modified Trypsin (Promega) and incubating at 37 °C for 3 h. After trypsin deactivation at 99 °C for 10 min and a cooling step, the digests (without the substrate) were added to Microcon DNA FastFlow membrane units (Millipore) and centrifuged for 20 min at 550 rcf. The flow-through (peptide fraction) was stored at −80 °C. For DNA recovery 20 µL of dH_2O were added, the membrane unit inverted and centrifuged for 3 min at 1000rcf. The DNA fraction was quantified using the Quantifiler Trio kit on a QuantStudio 5 Real-Time PCR System for DNA quantitation (QPCR). Short tandem Repeat (STR) typing used a Globalfiler kit (29 cycles) and a 3500 Genetic Analyzer with a 50 rfu analytical threshold (all Thermo Fisher Scientific Lifetechnologies).

Peptide calibrator samples for all five peptides were dissolved in buffer and mixed in equal concentrations at 0.05–50 nmol/mL. Run conditions on the Shimadzu LCMS-8050 mass spectrometer were established as follows. LC parameters: AdvanceBio Peptide mapping and guard columns (Agilent); flow: 0.50 mL/min; injection volume: 5 µL; temp: 55 °C; gradient: (mobile phase A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid), 0–25 min s, 15–65% B; 25–26 min s, 65–95% B. MS parameters: nebulizing gas: 2 L/min; heat and drying gas both: 10 L/min; interface temperature: 400 °C; DL temperature: 250 °C; heat block temperature: 400 °C; injection volume: 20 µL.

3. Results and discussion

All five signature peptides were successfully detected on the QqQ LC–MS with 2–3 target transitions each. Each peptide was present in different concentrations, with SEMG1 peptide 2 being the most concentrated and HIST being the lowest. As previously described peptide concentrations varied across donors [1]. Target transitions for the same peptide always displayed very close nmol values; this is a useful duplication step and helped identify an earlier non-specific signal. Detection was body fluid specific with no cross talk. The abbreviated 3 h trypsin digestion was compared to standard overnight digestion with all ten samples showing similar peptide concentrations. Deviating from Kranes et al [4] this assay includes an alkylation step with IAA and additional DTT denaturation prior to trypsin digestion after a comparison revealed higher detected peptide concentrations with IAA incubation. The signal intensities and body fluid coverage in the current multiplex are not well balanced. The HIST target is present in low concentrations and should be replaced. The addition of another semen marker would balance the number of protein targets for each body fluid.

To determine the limit of detection for authentic samples, liquid samples from the semen and saliva donors with the highest and lowest peptide concentrations were used to test 0.1 (1:10 dilution), 0.5 (1:2 dilution), and neat 1 and 2 µL amounts. For saliva, the more concentrated sample reached the lowest detection limit (LOD) with 1 µL of liquid sample for SMR3B-pep1 and 0.5 µL for HIST; for the lower concentrated samples these markers were only detected for 2 µL and SMR3B-pep2 had a LOD of 0.5 µL. For semen, the sample with the highest concentration of peptides was detected for all tested volumes, while for the less concentrated sample the LOD was reached at 0.5 µL for SEMG1-pep2. Other authors with higher resolution time of flight (TOF) mass spectrometers report much lower LODs with 1:1000 dilutions for saliva and 1:100,000 for semen [2,3]. This difference cannot be caused by donor to donor variation, we will explore ways to make the QqQ LC–MS assay more sensitive. All saliva or semen peptides were successfully detected for the eight different substrates and the 5-year-old semen samples with the exception of blue denim, where the HIST signal dropped out for saliva samples. Denim is generally known as a problematic substrate; in spite of the drop-out saliva would still have been identified based on the SMR3B signals.

Mixture study results on white cotton were as expected based on the different signature peptide concentrations in saliva and semen. The two semen markers were detected for all samples even as a minor component, while HIST and SMR3B-pep1 dropped out for the 2:1 (HIST) and 5:1 (both) mixtures. The same mixture series spotted on cotton swabs had different results. No HIST target ions were detected for the 1:1 mixture, all other samples had all expected signals present. The reason for this is unclear; more mixture series with these and more extreme ratios need to be tested to explore possible interference. Repeatability testing where the same analyst extracted and analyzed the same set of five sample three times, revealed a difference between saliva and semen. The relative standard deviation (RSD) was calculated over all signature ion concentrations in all three extractions: for semen this value ranged from 5 to 15%, while saliva peptides had a median RSD of 17% and a maximum value of 88%. Since pipetting and instrument variability would also have affected the semen samples, this difference must be sample type related. The saliva matrix may have an uneven distribution of targeted proteins. The repeatability study for the DNA fractions had a similar issue. All 30 fractions were quantified in duplicate; the RSD over the quantitation results varied from 7 to 89% for semen and 11 to 98% for saliva. This is most likely caused by extracted aliquots containing different amounts of cellular material. Overall DNA amounts were sufficient for STR typing and tested samples generated full profiles. Combining protein and DNA extraction preserves evidence and makes body fluid testing via signature peptide mass spectrometry more feasible. For semen evidence the assay would need to be combined with differential lysis. Semenogelin is associated with seminal fluid, not the sperm pellet. It should be possible to treat the epithelial cell fraction with trypsin to recover both DNA and the SEMG1 signature peptides.

4. Conclusions

We successfully adapted a QqQ LC–MS for signature peptides in body fluids. The semi-quantitative assay using an external standard determines nmol/mL concentrations for each target ion. Due to donor to donor variability this cannot be used to calculate back to initial body fluid amounts. Filtered three-hour trypsin digests were equivalent to overnight digested samples and did not require additional purification. Resulting DNA fractions were compatible with QPCR and STR typing with no indication of inhibition or degradation. Assay sensitivity was lower than for time of flight mass spectrometers [1,2]. The method is suitable for body fluid mixtures and is applicable for all stain substrates with no nonspecific signal. Future work will focus on adding additional semen and saliva markers, as well as increasing sensitivity. The method also shows promise for toxicology applications where signature peptide quantitation could serve as an internal marker for biological matrix variation.

Declaration of Competing Interest

None.

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