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Precise quantitation of 136 urinary proteins by LC/MRM-MS using stable isotope labeled peptides as internal standards for biomarker discovery and/or verification studies

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ABSTRACT

Spurred on by the growing demand for panels of validated disease biomarkers, increasing efforts have focused on advancing qualitative and quantitative tools for more highly multiplexed and sensitive analyses of a multitude of analytes in various human biofluids. In quantitative proteomics, evolving strategies involve the use of the targeted multiple reaction monitoring (MRM) mode of mass spectrometry (MS) with stable isotope-labeled standards (SIS) used for internal normalization. Using that preferred approach with non-invasive urine samples, we have systematically advanced and rigorously assessed the methodology toward the precise quantitation of the largest, multiplexed panel of candidate protein biomarkers in human urine to date. The concentrations of the 136 proteins span >5 orders of magnitude (from 8.6 μg/mL to 25 pg/mL), with average CVs of 8.6% over process triplicate. Detailed here is our quantitative method, the analysis strategy, a feasibility application to prostate cancer samples, and a discussion of the utility of this method in translational studies.

1. Introduction

Cancer is becoming increasingly prevalent in modern society [1,2]. While cardiovascular disease and lung/breast cancer are typically the most commonly discussed, cancers that affect proximal tissues of the urinary tract system (which includes the kidneys, ureters, bladder, prostate, and urethra) are also widespread [3]. To facilitate earlier detection and improved patient outcomes, methods for reliable and sensitive biomarker screening (through medical imaging or analyte biofluid monitoring, for example) must be developed and standardized. Typically, the untargeted biomarker discovery phase is performed on only a few samples, and this phase of the pipeline results in a large number of potential biomarkers that must be verified and validated on a larger number of samples before these markers can be used clinically [4].

Clinically preferred sample sources are minimally or non-invasive, with plasma and urine being most prominent. Urine – considered the ultrafiltrate of blood – is a popular biofluid for diagnostics due to its availability and ease of collection, as well as the high volume and regularity at which it can be obtained. With regards to sample processing techniques, immunoassays (conventionally ELISAs) are a preferred sample analysis technique due to their high sensitivity and sample throughput, but these can be prohibitive from a cost and development point of view [5]. This impacts their use in the discovery and verification phases of the protein biomarker pipeline, where hundreds to thousands of candidates need to be identified and screened. An attractive alternative for these assessment stages involves MS, which can be operated in an unbiased manner (as in data dependent acquisition on a hybrid ion trap-Orbitrap mass spectrometer [6]), a targeted manner (as in MRM on a triple quadrupole mass spectrometer [7]), or a semi-targeted manner (via parallel reaction monitoring on a

Abbreviations: AAA, amino acid analysis; ACN, acetonitrile; CV, coefficient of variation; CZE, capillary zone electrophoresis; DOC, sodium deoxycholate; DTT, dithiothreitol; FA, formic acid; GRAVY, grand average of hydropathy; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; NAT, natural; PC, prostate cancer; QA, quality assessment; QqQ, triple quadrupole; R^2, coefficient of determination; RPLC, reversed-phase liquid chromatography; SPM, single point measurement; SIS, stable isotope-labeled standard; TCEP, tris(2-carboxyethyl) phosphine; XIC, extracted ion chromatogram.

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hybrid quadrupole-Orbitrap mass spectrometer [8] or data independent acquisition on a hybrid quadrupole time-of-flight mass spectrometer [9]). Regardless of the platform, the method used for sample preparation should incorporate isotopically labeled standards (at the protein or peptide level) to help compensate for matrix-induced suppression and variation in instrument performance [10,11]. Additional benefits of the use of these standards are that they assist in correctly identifying the endogenous analytes, detecting chemical interferences, and in improving analytical precision [12].

Using the principles of "bottom-up" (*i.e.*, peptide-centric) liquid chromatography (LC)–MS/MS [13], a large number of qualitative techniques have been developed for protein biomarker identification in human urine. As indicated by many recent articles [14–23], and extensive reviews [3,24], identification of differentially expressed proteins has commonly been achieved through iTRAQ-based studies with multidimensional LC–MS/MS. Additional MS-based methods have also been developed using capillary electrophoresis [24] despite the lower mass loading capacity of the capillary columns compared to conventional LC columns [25,26]. Furthermore, CE has a volumetric limitation (low nanoliters vs. microliters in LC) that can also limit the sensitivity of MS detection if sample pre-concentration (via solid phase extraction, for instance) is not performed first. A smaller collection of studies, however, has focused on the quantitation of potential protein candidates in urine for a specific disease state [15,27–29]. In a recent example, Chen et al. quantified 63 proteins by multiplexed LC/MRM-MS, with 6 of these proving discriminatory for bladder cancer [27]. In fact, 3 of these 6 potential biomarkers were found to be over-expressed in their previous iTRAQ study [18], which highlights the utility of MRM in both discovery and verification research.

This current article describes our recently developed MRM-based method for quantifying a reproducible set of 136 proteins in human urine. The method utilizes a bottom-up reversed-phase liquid chromatography (RPLC)/MRM-MS workflow with a complex mixture of well-characterized SIS peptides. To simplify adaptation and ease of use, sample preparation/processing was performed without antibody-based depletion or enrichment and successive stages of LC fractionation. Preliminary developments centered on optimizing the sample pre-treatment, tryptic digestion, and chromatographic separation steps, as well as optimizing the MRM transitions of an enlarged panel of peptide surrogates toward the detection of more than 300 interference-free peptides. Endogenous protein quantitation was performed by using peptide standard curves under strict qualification criteria, with the subsequent results being rigorously assessed for reproducibility over process triplicate before exploring the feasibility of its application to diseased samples. Our recently developed Qualis-SIS software was used in this study, which greatly facilitated the analysis and quantitative interpretation of the results [30]. In addition to the description of the final method, the use of this tool on reference (or control) and patient (or diseased) urine samples is detailed here to provide a complete solution for evaluating and verifying highly multiplexed panels of potential markers of urological disease in future studies.

2. Methods

2.1. Urine collection and concentration

Pooled reference and individual patient urine was obtained from Bioreclamation (Hicksville, NY, USA), and was provided by consenting, de-identified human donors ($n = 12$ for reference and $n = 14$ for patient). The control urine donors were gender, age, and race matched to the male prostate cancer (PC) patients (65 years on average). The first voided urine was collected rather than other collection points (*e.g.*, second void, mid-stream, random spot) since this reportedly yields the lowest variability in protein concentration [31] and the largest percentage of prostatic secretions [28]. The urine samples were collected into sterile containers in the presence of an antimicrobial agent (sodium azide, 1 mM final), then aliquoted and distributed to us at the University of Victoria-Genome BC Proteomics Centre, where they were stored at −80 °C until analysis. Approval of this research was granted by the University of Victoria Research Ethics Board (protocol number: 13-095).

Prior to bottom-up processing, protein concentration and sample cleanup (which removes inorganic salts, sugars, cellular debris, etc.) was performed by centrifugal ultrafiltration, due to its reportedly superior performance compared to alternative cleanup methods, such as solvent-facilitated precipitation and equilibrium dialysis [31]. Ultrafiltration was performed with Amicon Ultra-4 centrifugal filters (10 kDa molecular weight cut-offs used in the final method; part No. UFC801096; Millipore; Bedford, MA, USA), in a manner that was in general accordance with the manufacturer's protocol. Briefly, after washing the semipermeable membrane with 4 mL of 25 mM ammonium bicarbonate, 4 mL of urine supernatant was passed through the concentrator at 4000×*g* and at ambient temperature. The concentrate was then successively washed with 4 mL of 20% acetonitrile (ACN) and 4 mL of water, both at 4000×*g* for 20 min. After lyophilizing the ultrafiltrate, the pellet was rehydrated with 25 mM ammonium bicarbonate and an aliquot was quantified for protein content by a bicinchoninic acid assay (product No. 23235; Pierce; Rockford, IL, USA). This was performed in order to estimate suitable loading amounts for such downstream processes as digestion, extraction, and separation.

2.2. Target panel and internal standards

The initial protein panel for this highly-multiplexed MRM-based assay was comprised of 494 unverified or undiscovered urinary biomarkers. With MS-based detection of target proteins being based on the detection of proteotypic peptides, target peptide selection for this method was governed by a set of rules that were designed to guide the synthesis of the isotopically labeled standards as well as enhance the digestion, separation, ionization, and detectability of both forms of the peptide (*i.e.*, the natural (NAT) and SIS) [32–34]. Included in the selection criteria were that the peptides have unique sequences within the human proteome, were previously observed in unbiased or targeted MS experiments, and were devoid of oxidizable residues (namely cysteine, methionine, and tryptophan). The latter is intended to help mitigate artifactual modifications during processing or storage, which could result in multiple forms of the peptide targets. The final quantitative panel comprises 136 proteins, with 213 interference-free peptides serving as the molecular surrogates. The reduction in panel size was due to peptides being either undetectable naturally, imprecise over inter-assay measurements, or possessing interference from co-eluting ions. The physicochemical properties (*e.g.*, isoelectric point, aliphatic index, grand average of hydropathy or GRAVY [35]) of these proteins and peptides were obtained from ExPASy's ProtParam tool [36].

The internal standards are isotopically labeled analogs of each endogenous peptide. Incorporation of the [^{13}C] and/or [^{15}N] (Cambridge Isotope Laboratories; Andover, MA, USA) isotopes was done at the C-terminal residue of tryptic peptides yielding mass shifts of +6 Da (from [$^{13}C_6$]-lysine), +8 Da (from [$^{13}C_6$, $^{15}N_2$]-lysine), or +10 Da ([$^{13}C_6$, $^{15}N_4$]-arginine) compared to their unlabeled counterparts. Peptide synthesis (performed recently on an Overture peptide synthesizer; Protein Technologies; Tucson, AZ,