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Multiplex and accurate quantification of acute kidney injury biomarker candidates in urine using Protein Standard Absolute Quantification (PSAQ) and targeted proteomics

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ABSTRACT

There is a need for multiplex, specific and quantitative methods to speed-up the development of acute kidney injury biomarkers and allow a more specific diagnosis. Targeted proteomic analysis combined with stable isotope dilution has recently emerged as a powerful option for the parallelized evaluation of candidate biomarkers. This article presents the development of a targeted proteomic assay to quantify 4 acute kidney injury biomarker candidates in urine samples. The proteins included in the assessed panel consisted of myo-inositol oxygenase (MIOX), phosphoenolpyruvate carboxykinase 1 (PCK1), neutrophil gelatinase-associated lipocalin (NGAL) and liver fatty acid-binding protein (L-FABP). The proteomic assay combined an antibody-free sample preparation and a liquid chromatography-selected reaction monitoring (LC-SRM) analysis pipeline. For accurate quantification of the selected candidates, we used PSAQ (Protein Standard Absolute Quantification) standards which are isotopically labeled versions of the target proteins. When added directly to the biological samples, these standards improve detection specificity and quantification accuracy. The multiplexed assay developed for the 4 biomarker candidates showed excellent analytical performance, in line with the recommendations of health authorities. Tests on urine from two small patient cohorts and a group of healthy donors confirmed the relevance of NGAL and L-FABP as biomarkers for AKI diagnosis. The assay is readily adaptable to other biomarker candidates and should be very useful for the simultaneous and accurate quantification of multiple biomarkers.

1. Introduction

Acute kidney injury (AKI) is a common and life-threatening condition with different causes including ischemia, sepsis or nephrotoxic substances. Clinical diagnosis of AKI is currently based on functional biomarkers, mainly serum creatinine, blood urea nitrogen and urine output characterized by a rapid decline in the glomerular filtration rate. Although widely used, these biological parameters provide little information on the underlying cause, the location and extent of kidney damage. In addition, serum creatinine is not sensitive to the loss of kidney reserve. To improve the specificity of diagnosis and detect kidney injury at early stages, intense efforts have been directed

Abbreviations: AKI, acute kidney injury; L-FABP, liver fatty acid-binding protein; MED-FASP, multiple enzyme digestion – filter aided sample preparation; MIOX, myo-inositol oxygenase; NGAL, Neutrophil gelatinase-associated lipocalin; PCK1, phosphoenolpyruvate carboxykinase 1; SRM, Selected Reaction Monitoring; PSAQ, Protein Standard Absolute Quantification

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to the development of novel biomarkers [1]. Several protein biomarker candidates were discovered in animal models of AKI and were subsequently evaluated in established human disease. Among these proteins, neutrophil gelatinase-associated lipocalin (NGAL), liver fatty acid-binding protein (L-FABP), kidney injury molecule 1 (KIM1) and interleukin-18 (IL-18) emerged as the most promising biomarkers for early detection of kidney injury [1,2]. However, none of these biomarkers obtained formal approval from health authorities for clinical use [3,4]. Recently, a clinical assay simultaneously quantifying insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinase 2 (TIMP2) in urine was approved by the Food and Drug Administration for use in patients at risk of developing AKI [5]. However, additional data from independent studies will be necessary before clinical certainty [3]. In the future, nephrologists will probably use combinations of biomarkers to diagnose specific AKI conditions (sepsis, cardiac surgery, toxic insult) [3]. In this context, high performance analytical tools allowing the simultaneous quantification of several biomarker candidates are necessary. Importantly, these tools must be compatible with small urine samples as AKI patients can be oliguric.

During the last decade, targeted proteomics based on liquid chromatography-selected reaction monitoring (LC-SRM) has emerged as a powerful alternative to immunoassays for the parallelized analysis of protein biomarker candidates in biofluids [6,7]. LC-SRM offers specific advantages including exquisite specificity, high sensitivity, high multiplexing capability and reproducibility [8]. In the field of nephrology, few recent studies described the development of LC-SRM assays for the clinical evaluation of putative AKI biomarkers [9–12]. Among these assays, the best multiplexing performance was obtained by Sigdel and coworkers [11]: 35 proteins were simultaneously quantified in urine, enabling the discrimination of the 3 major AKI phenotypes following kidney transplantation.

Targeted proteomics analyses based on LC-SRM are generally performed using a “bottom-up” workflow which involves the digestion of protein biomarker candidates into peptides and the targeted monitoring of signature peptides as candidate surrogates [13,14]. With this method, biomarker candidate concentrations can be determined using stable isotope-labeled standards (peptides, peptide concatemers or proteins) which are spiked into the samples and serve as references [15,16]. To meet the recommendations of health authorities for bioanalytical assay development, the use of PSAQ standards (Protein Standard Absolute Quantification) is advocated [17]. Indeed, because they are full-length isotope-labeled versions of the targeted proteins, PSAQ standards can be added to the biological samples at early stages of the analytical process and they can thus correct for analytical variabilities due to upstream sample handling or incomplete proteolysis (on the condition that they behave similarly to their protein targets during sample processing) [18–21].

The goal of this study was to develop a high performance proteomics pipeline, based on the use of PSAQ standards and LC-SRM, to simultaneously assay several AKI biomarker candidates in small urine samples. The pipeline was tested on extensively studied biomarker candidates, namely NGAL and L-FABP, and two new potential biomarkers selected from literature and expression data: myo-inositol oxygenase (MIOX) and phosphoenolpyruvate carboxykinase 1 (PCK1). MIOX expression is restricted to the proximal tubule epithelial cells [22]. It was recently identified as a potential plasma biomarker in human patients with AKI [23]. In the kidney, PCK1 is specifically expressed in the proximal tubule epithelial cells [22]. Based on this kidney-predominant expression, we hypothesized that PCK1 could leak into the urine following tubular necrosis. Results showed excellent analytical performance of the assay developed, and confirmed the utility of NGAL and L-FABP as biomarkers of AKI.

2. Material and methods

2.1. Urine samples

Urine samples from AKI patients were provided by nephrology departments from Henri Mondor Hospital (Créteil, France) and Tenon hospital (Paris, France). Experiments and research were conducted in accordance with the principles set out in the WMA Declaration of Helsinki. Urine samples were collected as part of clinical studies that were approved by ethical committee and declared at the Commission Nationale de l'Informatique et des Libertés. All patients provided written informed consent. Urine samples were collected, anonymized, rapidly aliquoted and stored at −80 °C. Patients were classified in two categories according to biopsy-proven pathological diagnosis: those with glomerular injury and those with tubular injury (Supplementary Table 1). Some biological samples were analyzed immediately at the clinical chemistry laboratory to determine standard parameters. Urine from healthy donors was also collected and used for analytical developments and to compare with AKI patients.

2.2. Recombinant proteins

Recombinant NGAL, PCK1 and L-FABP proteins were obtained from Abcam (references ab95007, ab119469 and ab82994 respectively). PSAQ standards (isotopically-labeled recombinant proteins) for the four biomarker candidates were synthesized as previously described [24]. Production was scaled-up at Promise Advanced Proteomics (Grenoble, France). PSAQ standards were checked for isotope incorporation (> 99%) and were quantified by amino acid analysis [25] (Supplementary Fig. 1).

2.3. Urine sample preparation

Urine samples were prepared based on an adaptation of the MED-FASP (multiple enzyme digestion – filter aided sample preparation) method [26]. Briefly, after thawing at room temperature, urine (400 µL) was spiked with defined amounts of PSAQ standards, gently mixed and centrifuged at room temperature for 10 min at 4000*g*. The supernatant was collected and concentrated to 100 µL on a 10-kDa cutoff ultrafiltration device (Amicon). Urinary proteins were denatured and reduced on the device in 4 M urea, 50 mM ammonium bicarbonate and 2 mM TCEP. The sample was washed twice with 4 M urea, 50 mM ammonium bicarbonate before performing alkylation in 4 M urea, 50 mM ammonium bicarbonate and 10 mM iodoacetamide. After two additional washing steps, the sample volume was reduced to 25 µL and proteins were digested for 3 h at 37 °C using trypsin/LysC mix (Promega, Charbonnières les Bains, France) at a protein/enzyme ratio of 1:30 (w/w). The urea concentration was reduced to 1 M and digestion was allowed to proceed overnight at 37 °C. Proteolytic peptides were recovered by adding 50 µL of NaCl 0.5 M to the filter and centrifuging for 40 min at 14 000g at room temperature. The peptide digest was purified on a C18 ZipTip device (Thermo Scientific, Courtaboeuf, France) and dried by vacuum centrifugation. Peptides were resolubilized in 10 µL of 2% acetonitrile, 0.1% formic acid, and 6 µL were injected into the LC-system.

2.4. Calibration experiment

Urine samples (400 µL each) were spiked with increasing amounts of surrogate analytes (unlabeled recombinant proteins) and constant amounts of PSAQ standards (20 ng/mL for PCK1, 30 ng/mL for NGAL and 10 ng/mL for FABP1). Zero samples were also constituted. The LLOQ was determined according to the FDA criteria described in the guidelines for bioanalytical method validation (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances). The LLOQ was established as the lowest concentration on