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Original Investigation

Urine Stability Studies for Novel Biomarkers of Acute Kidney Injury

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Background: The study of novel urinary biomarkers of acute kidney injury has expanded exponentially. Effective interpretation of data and meaningful comparisons between studies require awareness of factors that can adversely affect measurement. We examined how variations in short-term storage and processing might affect the measurement of urine biomarkers.

Study Design: Cross-sectional prospective.

Setting & Participants: Hospitalized patients from 2 sites: Yale New Haven Hospital (n = 50) and University of California, San Francisco Medical Center (n = 36).

Predictors: We tested the impact of 3 urine processing conditions on these biomarkers: (1) centrifugation and storage at 4°C for 48 hours before freezing at −80°C, (2) centrifugation and storage at 25°C for 48 hours before freezing at −80°C, and (3) uncentrifuged samples immediately frozen at −80°C.

Outcomes: Urine concentrations of 5 biomarkers: neutrophil gelatinase-associated lipocalin (NGAL), interleukin 18 (IL-18), kidney injury molecule 1 (KIM-1), liver-type fatty acid–binding protein (L-FABP), and cystatin C.

Measurements: We measured urine biomarkers by established enzyme-linked immunosorbent assay methods. Biomarker values were log-transformed, and agreement with a reference standard of immediate centrifugation and storage at −80°C was compared using concordance correlation coefficients (CCCs).

Results: Neither storing samples at 4°C for 48 hours nor centrifugation had a significant effect on measured levels, with CCCs higher than 0.9 for all biomarkers tested. For samples stored at 25°C for 48 hours, excellent CCC values (>0.9) also were noted between the test sample and the reference standard for NGAL, cystatin C, L-FABP and KIM-1. However, the CCC for IL-18 between samples stored at 25°C for 48 hours and the reference standard was 0.81 (95% CI, 0.66-0.96).

Limitations: No comparisons to fresh, unfrozen samples; no evaluation of the effect of protease inhibitors.

Conclusions: All candidate markers tested using the specified assays showed high stability with both short-term storage at 4°C and without centrifugation prior to freezing. For optimal fidelity, urine for IL-18 measurement should not be stored at 25°C before long-term storage or analysis.

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Development of novel biomarkers for early diagnosis, risk stratification, and prognosis of acute kidney injury (AKI) is a top priority in kidney research.[1] There are more than 100 published studies examining more than 20 novel urine biomarkers in various settings of AKI and chronic kidney disease (CKD).[2-4] Most studies do not measure novel biomarkers immediately upon collection. Instead, urine samples often are collected, processed, and stored under different protocols and subsequently assayed in batch. These protocols are based largely on opinion

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and convenience without evidence-based consensus about the optimal handling and processing of urine for evaluation.

The Assessment, Serial Evaluation, and Subsequent Sequelae in Acute Kidney Injury (ASSESS-AKI) Study is a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-sponsored multisite research consortium with the goal to examine the long-term outcomes of AKI.[5] A recent NIDDK workshop (Urine Biospecimen Handling Conference, February 22-23, 2010)[6] identified variations in storage and handling conditions as a major problem in using reposited samples. In response, we systematically examined how different real-world processing conditions might affect biomarker measurement, study interpretation, and eventual clinical application. The current standard for processing urine samples for clinical research studies is centrifugation and immediate storage at −80°C. However, in the clinical setting, samples may remain at room temperature or be refrigerated for several hours prior to processing with or without centrifugation. Therefore, we tested the effect of centrifugation and variations in short-term storage conditions on several promising urine biomarkers, including neutrophil gelatinase-associated lipocalin (NGAL),[7,8] interleukin 18 (IL-18),[9,10] kidney injury molecule 1 (KIM-1),[11,12] liver-type fatty acid–binding protein (L-FABP),[13] and cystatin C.[14,15]

METHODS

Patient Population

Hospitalized adult patients at the Yale–New Haven Hospital and University of California, San Francisco Medical Center were screened for eligibility. Each site targeted a different patient population. The New Haven site enrolled 50 unique intensive care unit patients admitted after cardiac surgery (coronary artery bypass graft surgery or valve replacement); biospecimens were collected within 48 hours of surgery. The San Francisco site enrolled 36 unique patients admitted from the emergency department to the intensive care unit or with a congestive heart failure exacerbation; biospecimens were collected as close as possible to the time of admission (for a description of the parent cohorts, see references[16,17]). Patients who were pregnant or nursing, were treated with long-term dialysis, had received a kidney transplant, or had a urostomy or nephrostomy were excluded from the study. At least 20 mL of urine was collected directly from the proximal reservoirs of a Foley catheter or a freshly voided clean-catch sample. All demographic and baseline information were obtained by medical chart review during the screening process. When ascertaining kidney function, the following definitions were used: AKI stage 1, 0.3-mg/dL or 50% increase in serum creatinine concentration during hospitalization; AKI stage 2, >100% increase in serum creatinine concentration from baseline during hospitalization; and CKD, estimated glomerular filtration rate <60 mL/min/1.73 m^2 using the CKD-EPI (CKD Epidemiology Collaboration) creatinine equation.[18]

The study was approved by the Institutional Review Boards of Yale University, Kaiser Permanente Northern California, and the University of California, San Francisco. All patients or their surrogates provided informed consent, with the exception of (1) patients who died before they or their surrogates could be approached for informed consent and (2) patients whose critical illness precluded them from providing informed consent and for whom a surrogate could not be identified after 28 days at the San Francisco site. For these 2 categories of patients, a waiver of consent was obtained.

Processing

The general approach is shown in Fig 1. Urine samples from each participant were divided and 10-mL aliquots were processed using the reference protocol (immediate centrifugation at 1,000*g* at 4°C for 10 minutes, followed by immediate aliquoting and storage at −80°C) and 1 of 3 test processes, including: process A, centrifugation followed by immediate aliquoting and temporary storage at 4°C for 48 hours prior to freezing at −80°C; process B, centrifugation followed by immediate aliquoting and temporary storage at 25°C for 48 hours prior to freezing at −80°C; and process C, samples not centrifuged but immediately aliquoted and stored at −80°C (Fig 1).

Fifty pairs of samples were dedicated to each condition tested. Some patients contributed to multiple conditions if sufficient urine was available.

Biomarker Analysis

Samples for each experimental condition and reference standard were frozen at −80°C for a median of 5 months before biomarker analysis. All biomarkers were measured in one batch. The laboratory and staff measuring the biomarkers were blinded to the study conditions.

Urine IL-18 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Medical & Biological Laboratories Co) per manufacturer's instructions (intra- and interassay coefficients of variation [CVs], 7.2% and 7.5%, respectively). The urine KIM-1 ELISA was constructed using commercially available reagents (Duoset DY1750; R & D Systems Inc) with intra- and interassay CVs of 5.6% and 4.9%, respectively. Urinary creatinine was measured by a modified Jaffé method. Analytical sensitivity of the creatinine assay is 0.05 mg/dL (intra- and interassay CVs, 0.6% and 1.1%, respectively). The urine NGAL ELISA was performed using a commercially available assay (NGAL ELISA Kit 036; Bioporto) that specifically detects human NGAL.[19] Intra- and interassay CVs for NGAL were 2.1% and 9.1%, respectively. Urine L-FABP was measured using a commercially available ELISA kit (CMIC Co) per manufacturer's instructions. Intra- and interassay CVs for L-FABP were 10.9% and 2.7%, respectively. Cystatin C was measured by latex particle-enhanced nephelometric immunoassay (PENIA) on a commercial nephelometer (Siemens). Intra- and inter-assay CVs for urine cystatin C were 5.2% and 4.8%, respectively.

Statistical Methods

The objective of the statistical analysis was to determine the agreement between measurements of urinary biomarkers between the reference standard and values obtained in samples prepared as process A, process B, and process C. Therefore, we calculated the concordance correlation coefficient (CCC),[20] for each scenario.[21] The CCC has a scale ranging between −1 (perfect negative agreement) and 1 (perfect agreement). Zero reflects no agreement. The CCC is a more appropriate statistic than the Pearson correlation coefficient for assessing the level of agreement between 2 measurements of the same item because the latter quantifies only the linear relationship, whereas the former quantifies the linear relationship under the assumption that the slope equals 1 and the intercept equals zero. In addition to calculating estimates of the CCC, we also calculated 95% confidence intervals (CIs). We required that the CCC must exceed 0.90 and 0.80 in order to claim