Impact of Hydrophobic Non-Specific Binding on Assay Recovery for Some Urinary Biomarkers of Acute Kidney Injury

Candace M. Adamo,[1]* Amar A. Sethi,[1] and Timothy H. Carlson[1]

Several sensitive low-abundance urine protein biomarkers have emerged for the diagnosis of acute kidney injury (AKI)[2], but preanalytical factors can negatively impact the accuracy of biomarker testing. In this study, we observed the effect of a hydrophobic nonspecific binding (HNB) inhibitor (ovalbumin) and protease inhibitors on the recovery of urine AKI biomarkers. The following 5 proteins were investigated: kidney injury molecule-1 (KIM-1) and retinol binding protein-4 (RBP-4), sandwich ELISA (R&D Systems); β_2-microglobulin (B2M; Siemens Healthcare Diagnostics), automated ligand-binding immunoassay (Immulite 2000); α_1-microglobulin (A1M; Kamiya Biomedical), automated immunoturbidimetric assay (Roche Modular P); and N-acetyl-β-D-glucosaminidase (NAG; Roche Applied Science), automated enzyme-activity method (Roche Modular P).

An initial study examined the impact of the presence and absence of ovalbumin and protease inhibitors (Oval+PI) on biomarker recovery in urine from 3 single donors. A1M and RBP-4 were not impacted by the addition of Oval+PI (recovery ranged from 95%–100% and 93%–105%, respectively). B2M, NAG, and KIM-1 were susceptible to diminished recovery in the absence of Oval+PI in a subset of the donors (recovery ranged from 31%–110%, 37%–100%, and 69%–83%, respectively).

To further investigate whether ovalbumin and protease inhibitors were both required, 3 urine pools were treated with either Oval+PI or ovalbumin alone. The same urine collection and treatment procedures were replicated on 2 consecutive days. On day 2, the pooled urine was spiked with recombinant KIM-1 and RBP4 to increase their concentrations. None of the other biomarkers were supplemented. Recovery of the biomarkers treated with Oval+PI compared to ovalbumin alone were consistent for all pools over the 2 days when treated with ovalbumin, regardless of whether PI was present (86.8%–127.2% recovery compared to ovalbumin alone).

This suggests that the protective effect of ovalbumin is not universal (i.e., recoveries of the biomarkers from some subjects were less impacted); however, urine from subjects that were impacted continued to be impacted in subsequent urine collections. In addition, recovery was relatively constant for both endogenous and recombinant proteins for KIM-1 and RBP-4 in all pools (86.8%–119.5%).

Next, biomarker recovery of an unpreserved pool treated with ovalbumin after thawing was compared to the same urine pool treated with

[1]Pacific Biomarkers, Seattle, Washington.
***Address correspondence to this author at:** 645 Elliott Avenue W., Suite 300, Seattle, Washington. Fax 206-298-9839; e-mail candacea@pacbio.com.
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[2] **Nonstandard abbreviations:** AKI, acute kidney injury; HNB, hydrophobic non-specific binding; KIM-1, kidney injury molecule 1; RBP-4, retinol binding protein; B2M, beta-2-microglobulin; A1M, alpha-1-microglobulin; NAG, N-acetyl-beta-D-glucosaminidase; Oval, ovalbumin; Oval+PI, ovalbumin plus protease inhibitors.

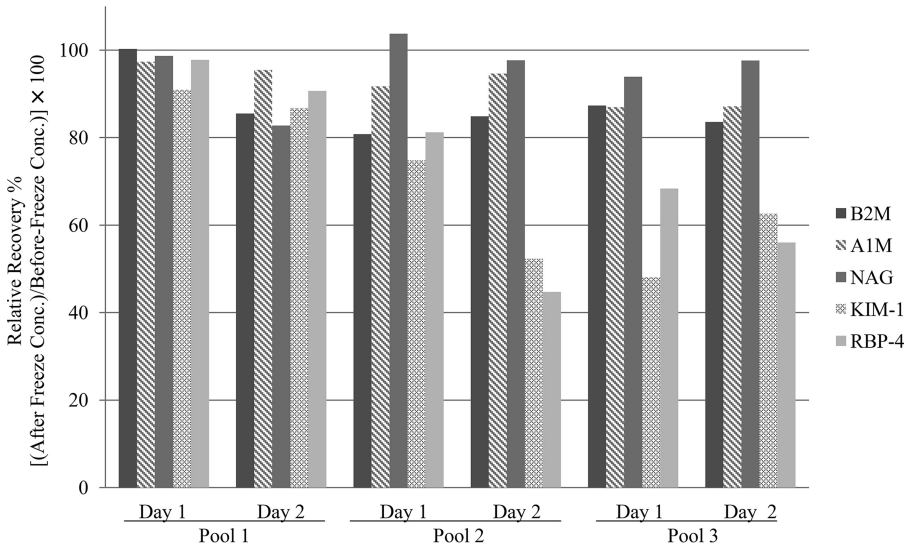

Fig. 1. Relative recovery of urine biomarkers with ovalbumin added before versus after freezing in 3 separate pools collected on 2 consecutive days.
Each column represents the biomarker's relative percent recovery in each urine pool (Relative Recovery % = (after-freeze concentration)/(before-freeze concentration) × 100; *n* = 3 pools containing urine from 3 individual donors).

ovalbumin before freezing (Fig. 1). Similar to the single donors, the addition of ovalbumin after thawing in pooled urine produced varying recoveries for KIM-1 and RBP-4 in a subset of urine pools (48.1%–91.0% and 44.7%–97.8%, respectively). The recoveries of B2M, A1M, and NAG were not impacted by the addition of ovalbumin, whether added before freezing or after thawing (80.8%–103.7% recovery). Overall, this study demonstrated that the addition of an HNB inhibitor (ovalbumin) improved recovery compared to no inhibitor and that the addition of protease inhibitors to the ovalbumin did not further improve recovery. Treatment of urine with ovalbumin before freezing can improve recovery of some low-abundance AKI biomarkers and potentially numerous other urine biomarkers. This suggests that accurate measurements of some of the novel AKI biomarkers investigated here require critical preanalytical handling before testing.

The magnitude of HNB is protein-specific and may be more critical when urine is stored in different types of glass or plastics (*1*). HNB has been prevented by several approaches, for example, by adding a detergent such as Tween 20 (*2*), Triton X-100 (*3*), or polyethylene glycol (*4*). However, these agents may affect enzyme activity or immunoturbidimetric assays, like the methods used for NAG and A1M in this report.

Proteases that could be present in urine may also contribute to the under-recovery of some biomarkers. However, when compared to the effect on recovery with ovalbumin plus a cocktail of protease inhibitors vs that of ovalbumin alone, no difference in the recovery of any of the biomarkers was observed. This does not completely rule out protease involvement because the higher levels of HNB inhibitor protein could act as a competitive protease inhibitor. An HNB inhibitor protein such as serum albumin may not be as good a substrate for proteases because of its very compact and extensive intramolecular disulfide bonding (*5*). Another reason to avoid

albumin is that albumin is commonly measured as part of assessing glomerular function in patients with kidney injuries.

This study demonstrates that some urinary biomarkers of AKI are susceptible to preanalytical losses, most likely resulting from nonspecific binding. In summary, treatment of urine with ovalbumin before freezing can improve recovery of some AKI biomarkers and potentially numerous other low-abundance urine biomarkers.

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