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SHORT REPORT

Overcoming the Effects of Matrix Interference in the Measurement of Urine Protein Analytes

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Abstract: Using multiplex bead assays to measure urine proteins has a great potential for biomarker discovery, but substances in urine (the matrix) can interfere with assay measurements. By comparing the recovery of urine spiked with known quantities of several common analytes, this study demonstrated that the urine matrix variably interfered with the accurate measurement of low abundance proteins. Dilution of the urine permitted a more accurate measure of these proteins, equivalent to the standard dilution technique when the diluted analytes were above the limits of detection of the assay. Therefore, dilution can be used as an effective technique for overcoming urine matrix effects in urine immunoassays. These results may be applicable to other biological fluids in which matrix components interfere with assay performance.

Keywords: biomarkers, body fluids urine, analysis/urine, standard addition, assay validation

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Introduction

The measurement of cytokines and other low abundance proteins using commercially available multiplex bead arrays could translate into new diagnostic or prognostic markers of disease.[1] However, the variability of urine matrix components such as organic compounds, pH and electrolytes can affect antibody binding and assay performance.[2] To account for these matrix effects, manufacturers of multiplex bead array systems have developed standard sample diluents for plasma, serum, cell culture and other biological specimens. No standard diluent has been developed for urine and many other biological fluids. Instead, manufacturers suggest that phosphate buffered saline be used as "urine-like" diluent. This approach does not account for the variability of matrix components in urine compared to more stable levels observed in plasma and serum.[3] Standard addition is a technique in which several different concentrations of an analyte being measured are added to the sample matrix. The total endogenous concentration of the analyte is calculated as the intercept from a plot of the signal responses of the detector against the spiked protein amounts. To improve the measurement of urine proteins using a fluorescence-based multiple bead assay, we conducted a series of experiments: (1) to determine the extent of matrix interference in urine and (2) to compare dilution versus standard addition methods for determining the unknown concentration of the proteins in urine samples.

Results

Recovery of proteins spiked in different urine samples

To determine the degree and variability of inhibition of protein measurement in urine, known concentrations of five proteins were spiked into urine samples from four patients with kidney disease (acute tubular necrosis, prerenal azotemia, and lupus nephritis) and wide variability in typical measurable matrix components (Fig. 1 and Supplemental Table 1). Standard curves for these five proteins were measured using a Bioplex multiplex bead array reader from Bio-Rad Laboratories Inc, using the Luminex 100 system according to the manufacturer's recommendations. Recovery of proteins was calculated as the percentage of interpolated protein concentration measured in the urine sample relative to the interpolated protein concentration in sample buffer. Resulting percentages reflect both the endogenous concentration of analyte and the matrix effect of the fluid. Recovery for the proteins in urine was highly variable (between 0.3 and 195% for MIP1α, for example), even between assays (Figs. 1 and 2). These results demonstrate that components of the urine matrix differ among urine samples and variably interfere with the accuracy of measurement of urine proteins in this assay. It is interesting to note that there is also variability in the effect of matrix between analytes.

Recovery of spiked proteins after dilution of urine samples

This experiment was designed to test the hypothesis that diluting urine will reduce the matrix effects on recovery of proteins. Urine samples were first diluted in sample buffer before measurement by bead array. In every case but one (MIP1α in urine from patient 1), dilution at 1:2 resulted in higher and more accurate measurement of the concentration of the spiked protein. Diluting unspiked urines at 1:20 and 1:10 in most cases resulted in the highest protein concentration measurements, suggesting that diluting matrix in the sample led to an attenuation of the matrix effect observed in an unspiked sample. For example, concentrations of IL6 and IL8 were 0.8 to 71 and 2 to 55 fold higher, respectively, in diluted than in the undiluted samples.

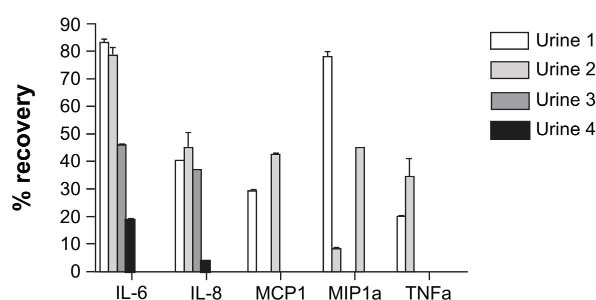

Figure 1. Recovery of five proteins spiked into urine samples from patients with kidney disease. Four different urine samples from patients with acute tubular necrosis, prerenal azotemia, and lupus nephritis were spiked with known quantities of each of five analytes. The percent recovery of analyte was calculated by subtracting the measured concentration of unspiked urine from the measured concentration of urine spiked with standard then dividing by the expected concentration of that standard (([measured urine$_{spiked}$] − [measured urine$_{unspiked}$])/[expected standard] × 100).
Note: Results were reported as means ± standard deviations.

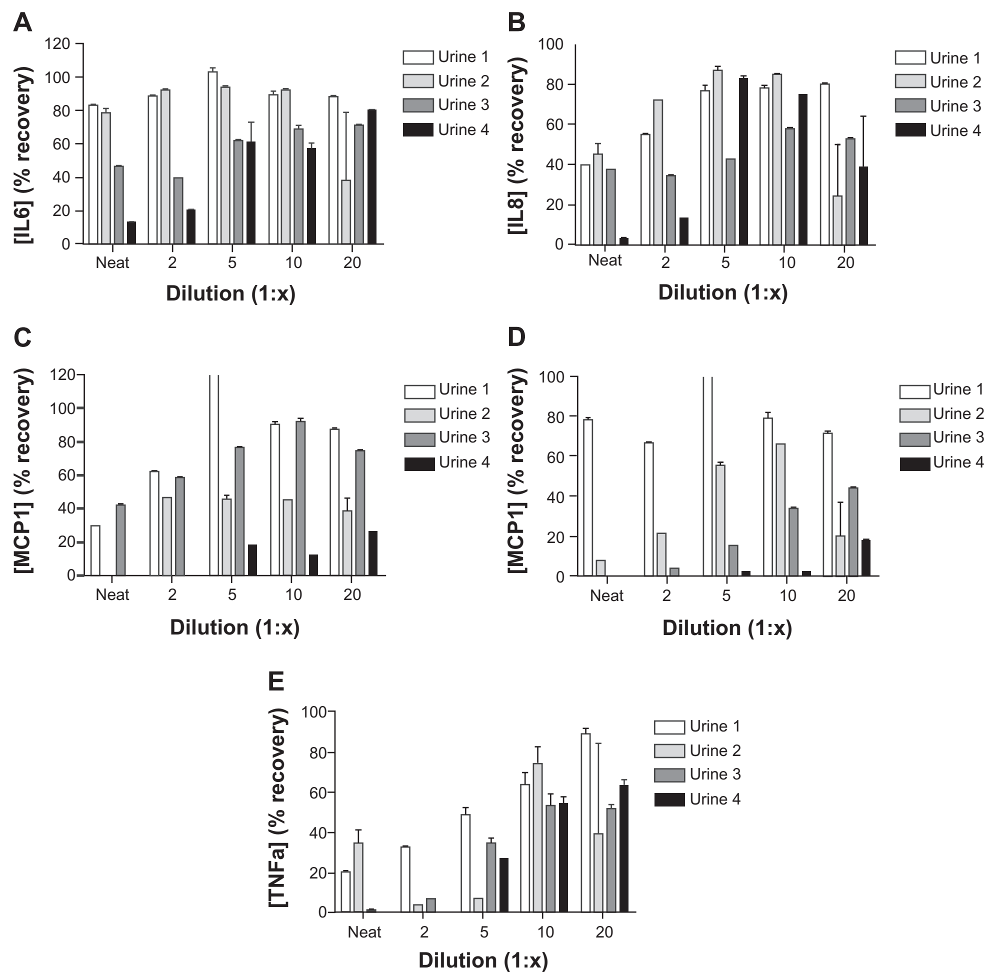

Figure 2. Recovery of analytes spiked into urine samples and after dilution. Urine samples from the same four patients in Figure 1 were diluted in sample buffer at either neat concentrations or 1:2, 1:5, 1:10 and 1:20. Samples were then spiked with known amounts of five different protein analytes. The percent recovery was calculated using the formula from Figure 1.
Notes: Results were reported as means ± standard deviations.

Some diluted samples did not have concentrations higher than those in the undiluted samples, but for many of these the concentration measured was so low that diluted concentrations were near the limit of quantification. This suggests that overcoming the matrix effect with dilution in samples with very low levels of endogenous analyte was ineffective because concentrations were diluted to below the limit of quantification of the assay (examples include MCP1 in urines 2 and 4 and MIP1α in urines 3 and 4). Similarly, the percent recovery of protein after it was spiked into diluted samples tended to be best at the higher levels of dilution (1:10 or 1:20). This effect was pronounced in IL8, MIP1α and TNFα (Fig. 2). Therefore, diluting urine appears to be an effective way to overcome individual matrix effects of different urine samples.

Standard addition for determining protein concentrations and comparison with concentrations determined in diluted samples

To evaluate the values of concentration obtained by dilution, we compared them to the gold standard method for determining concentrations in an inhibitory matrix–standard addition.[4] A standard addition plot was created by spiking urine samples with six known concentrations of analyte and plotting the concentration of spiked analyte on the x-axis and the corresponding fluorescence intensity on the y-axis. A line was then fitted to the plot and the unknown concentration was then determined as minus the estimated x-intercept (Fig. 3). The resulting concentrations determined by this analysis were compared to the concentrations of the unspiked

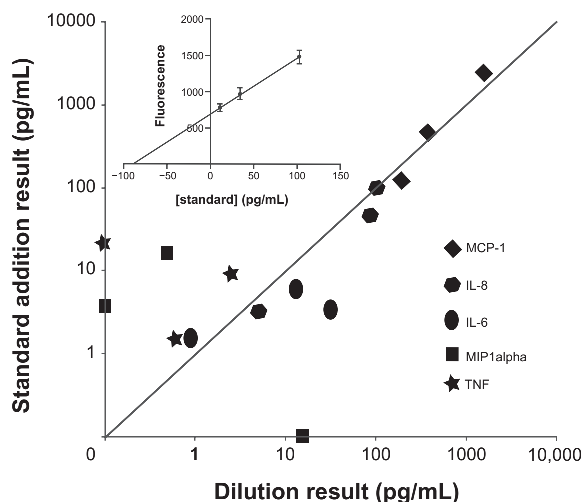

Figure 3. Comparison of dilution and standard addition techniques for overcoming matrix effects. Analyte concentrations from the same four urine samples from Figures 1 and 2 were measured by diluting each sample 1:10 and by using the standard addition technique. Standard addition was performed by spiking several concentrations of analyte into neat urine. The inset is an example of how the standard addition technique is used to determine the concentration of an unknown sample. The analyte concentration is calculated as the x-intercept multiplied by –1. A line of identity demonstrates how closely (when the diluted concentration of analyte was above the limit of quantification) the dilution and standard addition techniques determine analyte concentration for each of the four urine samples.
Note: Results were reported as means ± standard deviations.

samples diluted 1:10 (Fig. 3). This dilution was used because it provided the most consistent restoration of recovery (Fig. 2) without further dilution of sample that could result in levels below the limit of quantification of the assay. An identity line was shown to highlight the similarities in the concentrations obtained for the two techniques, ie, the nearer a point to the identity line, the more similar the two measured concentrations. The two techniques achieved greater agreement for analytes with higher concentrations than for those on the lower end of the concentration range (around 50 pg/ml or less), suggesting that measuring proteins using a dilution method can be effective for overcoming matrix effects in urine when concentrations are above the lower limits of detection.

Discussion

This study demonstrates a large amount of variability in protein measurements due to the effects of the urine matrix. Concerning is that both assay and urine elements appear to contribute to this variability. The difference in recovery between analytes observed in Figure 1 may relate to matrix components masking the antibody or analyte epitopes.[5] This effect may be differential based on the charge and 3-dimensional structure of the variable region of the antibody or of analyte epitopes. In our study, the matrix interference did not disappear with changing the pH or osmolality of the sample, with addition of milk as a blocking agent, or with removal of low molecular weight matrix elements with spin columns (data not shown). This has important implications for validation of immunoassays to measure low abundance proteins in the urine. Before measures can be considered validated, assays for all analytes in a multiplex bead array must be tested for recovery of spiked standards and the effectiveness of dilution as a method for reducing matrix effects. If this method is not effective or if analytes are below the limit of detection of the assay, standard addition must be used. Similar to our findings, a comprehensive international study in 12 laboratories using 14 different immunoassays (including Luminex-based bead arrays) found that the matrix effects of vaginal mucosal fluid, serum, and saline on recovery of IL1β and IL6 were large and variable across assays and laboratories,[2] demonstrating that the problem of biological fluid matrix elements reducing analyte recovery is not isolated to urine. Urine is a similarly complex fluid with a large variation in matrix content, and it is difficult to determine the specific components of urine that may interfere with the assay. More applicable to this study, Wood et al similarly advocated the use of dilution to measure IL6 in urine.[6]

Standard addition is a well-established approach for overcoming matrix effects.[4,7] However, standard addition is time consuming and requires a larger number of measurements per sample. In these experiments, sample dilution was effective when endogenous protein concentrations were well above the limit of quantification. Nonetheless, when urine analytes are present at concentrations close to the lower limit of quantification of the assay, standard addition should be used to determine concentrations. This would suggest that dilution would be an effective means of determining analyte concentration in urine when the assay cutoff between cases and controls in any given study is well above the lower limit of quantification. These findings likely apply to other biological fluids

with matrix components that interfere with assay performance.

Methods

Urine analyte analysis

Urine protein concentrations were measured using commercially available multiplex human cytokine assays and a Bioplex multiplex bead array reader from Bio-Rad Laboratories Inc, (Hercules, CA) that uses a Luminex 100 system (Luminex Corp., Austin, TX). As per manufacturer's suggestion, a diluent containing PBS (pH 7.4) and 0.5% BSA was used to prepare the standards. Prior to analysis, the bead array reader was calibrated per manufacturer's instructions. Urine proteins from premixed kits (Bio-Rad) analyzed were IL6, IL8, monocyte-chemoattractant factor-1 (MCP1), macrophage inhibitory protein (MIP1)α and tumor necrosis factor (TNF)α. All results were expressed as means of four replicate values. Limits of quantification were defined as the lowest and highest concentration at which the percent coefficient of variation were below 10%. The interpolated lower limits of quantification for IL6, IL8, MCP1, MIP1α, and TNFα were 1, 9, 13, 8, and 8 pg/ml respectively.

Recovery of proteins spiked into urine samples

Four urine samples with a large degree of variation in typical measurable components were chosen as test samples (Na, <10 to 106 mM; K, 8 to 44 mM; Ca, <2 to 6.5 mg/dl; UUN, <50 to 1406; Osm, 252 to 629 mOsm; pH, 4.6 to 7.8; measured by the Medical University of South Carolina clinical laboratory; Supplemental Table 2). Each urine sample was spiked with a known quantity of the protein standards within the measurable range of the standard curve (IL6, 1046; IL8, 588; MCP1, 679; MIP1α, 567; TNFα 2294 pg/ml). Each analyte and each unspiked and spiked urine sample were assayed in quadruplicate and averaged.

Recovery of proteins after a series of dilutions with standard diluent

A series of dilutions of the four urine samples used in experiment 1 were made by diluting with the diluent used to create the standard curve (PBS/0.5% BSA). Samples diluted 1:20, 1:10, 1:5, and neat were analyzed in quadruplicate and averaged. Then, samples first diluted 1:20, 1:10, 1:5, 1:2 and neat were spiked with standard (IL6, 860; IL8, 693; MCP1, 687; MIP1α, 424; TNFα, 2057 pg/ml) and analyzed in quadruplicate and averaged.

Standard addition for determining protein concentrations

An experiment was designed using standard addition as previously described[7] to determine the unknown concentrations of proteins. Urines 1, 2 and 3 were spiked with five levels of standard (2743 to 7 pg/ml), and these and an unspiked sample were analyzed in quadruplicate. Regression lines for the points generated for each urine and each analyte (added concentration of standard—x-axis; instrument response—y-axis) were created using the resistant least trimmed squares method (function ltsreg in the MASS package)[8] for R.[9]

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Disclosures

Mr. Taylor has no financial interests to disclose.

Dr. Oates has no financial relationships relevant to this publication to disclose. However, he has had in the last year been a speaker for Abbott Laboratories. He has received research samples and data from Genentech and Bristol Meyers Squibb. He receives funding to conduct clinical trials from Teva, Human Genome Sciences, and Cephalon.

Author(s) have provided signed confirmations to the publisher of their compliance with all applicable legal and ethical obligations in respect to declaration of conflicts of interest, funding, authorship and contributorship, and compliance with ethical requirements in respect to treatment of human and animal test subjects. If this article contains identifiable human subject(s) author(s) were required to supply signed patient consent prior to publication. Author(s) have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have consent to reproduce any copyrighted material. The peer reviewers declared no conflicts of interest.

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Supplemental Methods for Standard Addition

For each urine and each analyte, the estimates of the slope and intercept were obtained by minimizing the 13 (for our data) smallest squared residuals, and the estimated concentration was obtained as the corresponding x-intercept. This resistant method was selected following comparison with ordinary least squares, robust least squares (function rlm in the MASS package), and a Tukey-based method (where data points with residuals classified as outliers were omitted.[1] By applying the method to the full data and again to data excluding the raw sample, a percent recovery for the smallest spike amount was determined, which, for the resistant method, proved more robust to subject variability for more analytes (see Supplemental Fig. 1).

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Table S1. Diagnosis and renal function in patients 1–4.

Patient #	Diagnosis	Renal function surrogate
90 (1)	Acute tubular necrosis	Serum Cr 6.0
414 (2)	Prerenal azotemia	Serum Cr 1.8
918 (3)	Prerenal azotemia	Serum Cr 2.2
923 (4)	Class V lupus nephritis	Serum Cr 5.1

Table S2. Urine components for individual patient urine samples.

	Urine 1 (90)	Urine 2 (414)	Urine 4 (923)	Urine 3 (918)	PBS [0.5%BSA]*
Ca (mg/dl)	6.5	< 2	2	2	0
Ucr (mg/dl)	15	112	210	90	0
K (mM)	7.7	44.2	30.3	24.8	2.7
Na (mM)	106	74	14	< 10	138
UUN (mg/dl)	< 50	975	348	1406	0
Osm	252	549	273	629	270
pH	7.8	6.7	4.6	5.3	7.4

*Standard diluent, calculated values urines were measured by the central Lab at the Medical University Hospital.

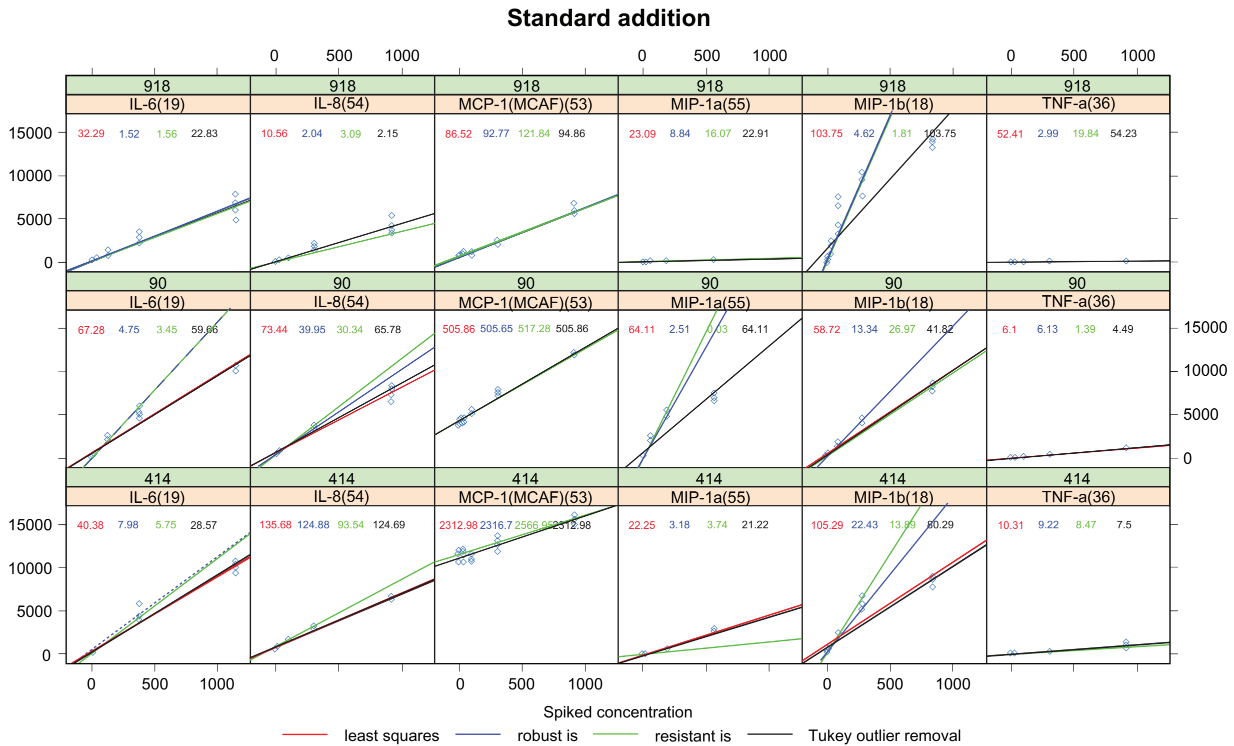

Figure S1. Fitted lines for each of the 4 methods for each subject's data (for each protein). Also shown in each panel are the concentrations for the raw urine sample derived using standard addition obtained by extrapolating each of the fitted lines.
Abbreviation: ls, least squares.

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