Technical Note

Urine protein concentration estimation for biomarker discovery

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Abstract

Recent advances have been made in the study of urinary proteomics as a diagnostic tool for renal disease and pre-eclampsia which requires accurate measurement of urinary protein. We compared different protein assays (Bicinchoninic acid (BCA), Lowry and Bradford) against the 'gold standard' amino-acid assay in urine from 43 women (8 non-pregnant, 34 pregnant, including 8 with pre-eclampsia. BCA assay was superior to both Lowry and Bradford assays (Bland Altman bias: 0.08) compared to amino-acid assay, which performed particularly poorly at higher protein concentrations. These data highlight the need to use amino-acid or BCA assays for unprocessed urine protein estimation.

Keywords: Protein concentration assays, proteomics, urine.

Background

Protein excretion in urine is associated with many pathologies including the pregnancy specific syndrome pre-eclampsia. Characterising specific proteins in urine is now achievable through advances in proteomic technologies and the use of urine as a source of candidate biomarkers and therapeutic targets is rapidly developing. Recently proteomic techniques have identified potential diagnostic and predictive urinary biomarkers for pre-eclampsia [1-4].

Urine protein estimation of different clinical laboratory techniques have previously been tested but this has not been completed for standard research methods [5]. Proteomic analysis requires precise assessment of total protein concentrations to enable accurate quantitation by subsequent downstream gel-based and tandem mass spectrometry (MS/MS) [6], and is a requisite to confidently explore the role of future biomarkers.

Whilst there is a move to standardise urine collection for urinary proteomic assessment by the Human Human Kidney and Urine Proteome Project (HKUPP) and the European Kidney and Urine Proteomics (EuroKUP) networks (www.hkupp.org; www.eurokup.org;), publications on urinary proteomics use a variety of assays to estimate total protein concentrations (e.g. Bradford and Coomassie Plus assays,[7-11] BCA)[12, 13] or assays are not defined. However, these tests were not specifically developed to quantify protein in urine and may suffer inaccuracies due to interference by urinary solutes or pH. High urea concentrations are also likely to interfere with Bradford assay due to the incompatibility of coomassie based protein assays to surfactants, e.g. urea, even at low concentrations, causes precipitation of the reagent [14].

The objective of this study was to assess which protein assay provided the most accurate quantification across a wide range of urinary protein concentrations. We performed three standard assays (Bicinchoninic acid (BCA), Lowy and Bradford) and compared these to the current gold standard amino-acid assay.

Methods

Sample collection

Urine samples with a diverse range of protein concentrations were collected from healthy pregnant women at 15 weeks' (n = 12) and 20 weeks' (n = 12) gestation. Urine samples were also collected from women who had been diagnosed with pre-eclampsia (n = 8); according to International Society of Study of Hypertension in Pregnancy Guidelines[15] and from healthy non-pregnant women of reproductive age (n = 8). All collections were approved by the St. Thomas' Local Ethics Committee (09/H0802/031) and obtained following informed written consent. Once collected, urine samples were centrifuged at 1400 x g for 10 minutes at 4^{o}C and then stored in aliquots at -80^{o}C until required for protein concentration assays.

Protein estimation of urine

Prior to protein concentration assays, urine aliquots (1.8 ml) were ultracentrifuge concentrated to approximately 170 µL using a 3,000 MW filtration column (Millipore Centrifugal Filter Units). Protein concentration was first estimated using the amino acid assay. Subsequent assays using the Lowry, BCA and Bradford assays (Thermo Scientific) were then performed on the same urine samples following manufacturers' protocols after urine dilutions for each sample set and assay were optimised to fit within the recommended standard curve concentration ranges.

Statistical analysis

Initial visual analysis was completed by scatter plots comparing each of the three protein assays with the amino acid assay. The amino acid assay results were then compared to the other assays using the Bland-Altman method. For each pair of measures, the average and difference were calculated; and 95% reference ranges defined as the mean of the differences ± 1.96 x SD. The closer the reference range is to zero, the closer the agreement between the methods of measurement [16].

Results

Of the three spectrophotometric assays tested, the BCA compared closest to amino acid analysis for determining the protein concentration in urine (Fig. 1a). Furthermore, the Bland Altman test indicated the BCA assay produced similar results to the gold standard amino acid assay (Table 1 and Figure 2). Both the Bradford and Lowry assays greatly underestimated the protein concentration in the urine samples. There were no differences in the performance of assays between non-pregnant and pregnant urine samples, indicating the BCA assay in particular has utility for determining urinary protein concentrations in both pregnant and non-pregnant conditions.

We conducted an evaluation comparing the standard laboratory assays against the gold standard amino acid assay. Samples from pregnant and non-pregnant women were used to ensure that a range of protein concentrations were assessed. The BCA performed considerably better and outperformed the Lowry and Bradford assays.

Discussion

Urine is an attractive source of potential clinical biomarkers due to the large quantities available and non-invasive nature of collection. Proteomic analysis not only gives insight into biological processes within the kidney and urogenital tract, but due to glomerular filtration of a subject's blood, circulating biomarkers may also be identified. The complexity of the urinary proteome is rapidly evolving with the development of MS based approaches and over 3500 proteins have now been isolated detected as excreted under different conditions [6, 11, 13, 17].

Although urine is readily available in large quantities, its low and variable protein concentration, salt content and other potential contaminants make multiple purification and preparation steps necessary for proteomic analysis. Whilst there has been considerable progress in exploration and standardisation of urine sample collection [18] and downstream analytical methodologies, reviewed by ourselves [6],

and there is a paucity of data regarding which bench protein assay is most appropriate for urine protein quantification. Due to the multiple steps required prior to proteomic analysis a great deal of variability may be introduced, which may compromise validity of results, particularly those assessing biomarker quantification. As one of these steps involves assessment of total protein concentration in each urine sample in order to process the desired amount of protein for mass spectrometric analysis, an accurate reproducible protein assay is fundamental to every urinary proteomic workflow.

The amino acid assay is considered the gold standard for protein quantification; however it is both expensive and time consuming, therefore for routine urinary protein quantification assay that may be performed in a routine laboratory setting is desirable.

In conclusion, we have identified the BCA protein assay to be a suitable alternative to the amino acid analysis gold standard for the accurate assessment of total protein in urine samples. We recommend it as a rapid technique that can be performed in the local laboratory environment for all urinary proteomic workflows, to reduce inherent variability in protein concentration estimates and enable more robust quantitative proteomic analysis.

Competing Interests: The authors declare that have no competing interests.

Authors' contributions: HDM and AJW carried out the protein assays. HDM and KB consented and collected the urine samples from participants. HDM, LCC, AJT and MAW conceived the study, and participated in its design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

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Table & Figure legends

Table 1: Bland-Altman bias and limits of agreement for all assays compared to the amino acid assay

Figure 1: Scatter plots comparing the amino acid assay (AAA) with a) BCA; b) Bradford assay; and c) Lowry assay. Dashed reference lines are y=x.

Figure 2: Bland-Altman plot comparing the amino acid assay (AAA) with the BCA indicating that the BCA method is most comparable.

Table 1:

Assay Comparison	**Bias**	**95% Limit of Agreement**	
		From	**Till**
Bradford Assay vs. Amino Acid Assay	-1.7	- 6.9	3.5
BCA vs. Amino Acid Assay	0.08	-0.7	0.9
Lowry Assay vs. Amino Acid Assay	-2.4	-7.0	2.3