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1 **Technical Note**

2 **Urine protein concentration estimation for biomarker discovery**

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27 **Abstract**

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

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38 **Keywords:** Protein concentration assays, proteomics, urine.

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42 **Background**

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

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

53 Whilst there is a move to standardise urine collection for urinary proteomic assessment by the Human
54 Human Kidney and Urine Proteome Project (HKUPP) and the European Kidney and Urine Proteomics
55 (EuroKUP) networks (www.hkupp.org; www.eurokup.org), publications on urinary proteomics use a
56 variety of assays to estimate total protein concentrations (e.g. Bradford and Coomassie Plus assays,[7-
57 11] BCA)[12, 13] or assays are not defined. However, these tests were not specifically developed to
58 quantify protein in urine and may suffer inaccuracies due to interference by urinary solutes or pH. High
59 urea concentrations are also likely to interfere with Bradford assay due to the incompatibility of
60 coomassie based protein assays to surfactants, e.g. urea, even at low concentrations, causes precipitation
61 of the reagent [14].

62

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

67

68 **Methods**

69 *Sample collection*

70 Urine samples with a diverse range of protein concentrations were collected from healthy pregnant
71 women at 15 weeks' (n = 12) and 20 weeks' (n = 12) gestation. Urine samples were also collected from
72 women who had been diagnosed with pre-eclampsia (n = 8); according to International Society of Study
73 of Hypertension in Pregnancy Guidelines[15] and from healthy non-pregnant women of reproductive
74 age (n = 8). All collections were approved by the St. Thomas' Local Ethics Committee (09/H0802/031)
75 and obtained following informed written consent. Once collected, urine samples were centrifuged at
76 1400 x g for 10 minutes at 4°C and then stored in aliquots at -80°C until required for protein
77 concentration assays.

78

79 *Protein estimation of urine*

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

86

87 *Statistical analysis*

88 Initial visual analysis was completed by scatter plots comparing each of the three protein assays with
89 the amino acid assay. The amino acid assay results were then compared to the other assays using the
90 Bland-Altman method. For each pair of measures, the average and difference were calculated; and
91 95% reference ranges defined as the mean of the differences $\pm 1.96 \times \text{SD}$. The closer the reference
92 range is to zero, the closer the agreement between the methods of measurement [16].

93

94

95 **Results**

96 Of the three spectrophotometric assays tested, the BCA compared closest to amino acid analysis for
97 determining the protein concentration in urine (Fig. 1a). Furthermore, the Bland Altman test indicated
98 the BCA assay produced similar results to the gold standard amino acid assay (Table 1 and Figure 2).

99 Both the Bradford and Lowry assays greatly underestimated the protein concentration in the urine
100 samples. There were no differences in the performance of assays between non-pregnant and pregnant
101 urine samples, indicating the BCA assay in particular has utility for determining urinary protein
102 concentrations in both pregnant and non-pregnant conditions.

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

107

108 **Discussion**

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

115

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

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

126

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

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

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

138

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

143

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218 **Table & Figure legends**

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

220 **Figure 1:** Scatter plots comparing the amino acid assay (AAA) with a) BCA; b) Bradford assay; and c)

221 Lowry assay. Dashed reference lines are $y=x$.

222 **Figure 2:** Bland-Altman plot comparing the amino acid assay (AAA) with the BCA indicating that the

223 BCA method is most comparable.

224

225 **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

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