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Problems with the estimation of urine protein by automated assays

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Abstract

Objectives: Most clinical laboratories replaced their manual precipitation techniques for the determination of urinary protein with automated dye binding assays or benzethonium chloride-turbidimetric assays. Few studies have validated these assays for the measurement of urinary proteins in the normal range.

Design and methods: This study compares four automated assays for the measurement of urinary protein to a manual Ponceau S/TCA precipitation assay. We evaluated the linearity, the precision, the analytical sensitivity, the accuracy and the recovery of different proteins for each assay.

Results: All assays showed good linearity with the theoretical concentration of albumin present in the sample. The coefficient of variation was below 10% at a concentration of 0.142 g/L. However, the manual Ponceau S/TCA assay demonstrated superior analytical sensitivity. Accuracy determinations showed a variable positive bias and poor correlations at concentrations below 0.1 g/L when compared to the Ponceau S/TCA assay. Small molecular weight peptides particularly affected the pyrogallol red assays but other urinary components also interfered with the automated assays.

Conclusions: Most automated assays show high imprecision and poor accuracy for the measurement of urinary protein in the normal range. The Ponceau S/TCA offers a precise and accurate manual alternative to these automated assays.

Keywords: Urine; Protein; Measurement; Automated; Comparison; Bias; Precision; Linearity

Introduction

Normal urine proteins consist of albumin, Tamm–Horsfall protein, fragments of immunoglobulins, and low molecular weight proteins [1,2]. Normal values for 24-h urinary excretion of total proteins vary between 100 and 200 mg, depending on the assay used and the population that was studied [2–4]. Proteinuria is generally considered normal when it is below 150 mg/day in healthy adults. The expected reference ranges found in the kit inserts from Beckman, Ortho Diagnostics, Randox and Roche show this variation and are <100 mg/24 h, <230 mg/24 h, <141 mg/24 h, and <150 mg/L (no 24-h value in the insert), respectively. Most of the reports for the establishment of these reference values were produced before 1990. The methods used then were mainly precipitation assays with turbidimetric or nephelometric determinations and dye binding assays were used by only a minority of laboratories. Today, most laboratories use direct dye binding assays or automated turbidimetric assays and few studies have established reference values with these methods.

We have repeatedly observed discrepancies between urinary protein determinations by some automated analyzers and the expected agarose electrophoresis band intensities. This led us to believe that the urinary protein assay we were using might be biased by certain constituents of the urine not visible on electrophoresis. The aim of this study was to compare the TCA–Ponceau S precipitation

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assay with four automated urinary protein assays. We evaluated the linearity, the analytical sensitivity, and the recovery of albumin, gamma-globulin, a protein mixture (albumin, globulins and paraprotein), Tamm–Horsfall protein, and polypeptides with a molecular weight (MW) <10,000 Da. Furthermore, we evaluated the recovery of albumin at low protein concentrations in order to assess the assay performance in a normo-proteinuric population.

Materials and methods

Working materials

Purified human serum albumin (sterile 25% solution in saline) was obtained from Alpha Therapeutic Corporation, Los Angeles, CA. This stock solution was used for the preparation of different albumin concentrations and for calibration. Purified human gamma-globulins (Cohn fraction II and III) were obtained from Sigma, St. Louis, MO. A stock solution of gamma-globulins of 50 mg/mL was prepared and used for further experiments.

Tamm–Horsfall protein was crudely purified from normal urines by 1.0 M NaCl precipitation followed by centrifugation at 5000 × *g* for 20 min [5]. The supernatant was removed and the remaining solution was transferred into 50-mL conical tubes and centrifuged at 5000 × *g* for 30 min. The supernatant was again removed and the crude protein preparation was diluted 1:2 with sterile water. The concentration of protein was estimated by the Beckman serum protein assay (Biuret derivative) and SDS electrophoresis confirmed the presence of a 93-kDa protein (>90%).

The protein mixture was obtained from a urine sample with a proteinuria characterized by a glomerular pattern and a Bence–Jones protein. The different protein fractions were quantified by electrophoresis (49.9% albumin, 21% alpha-1, 15.8% alpha-2, 11.2% Bence–Jones and 2.2% gamma). A polypeptide solution was prepared from a casein hydrolysate obtained from bovine milk (N-Z-Amine EKC powder, Sigma, St. Louis, MO). Briefly, 10 g of powder was mixed for 30 min with 50 mL of normal saline. This solution was centrifuged at 3000 × *g* for 30 min, then the supernatant was retrieved and was filtered on a 10-kDa molecular weight filter (Vivascience, Westford, MA). The filtrate was collected and assayed for total protein by the Beckman serum protein assay (Biuret derivative).

Urinary protein measuring methods

Protein determination by the TCA–Ponceau S method was adapted from Pesce and Strande [6]. Briefly, Ponceau S and TCA stock solutions were prepared by dissolving 4 g of Ponceau dye (Sigma, St. Louis, MO) in 100 mL of distilled water and 30 g of TCA in 100 mL of distilled water. A working reagent with a concentration of 120 mg/L of Ponceau S and 4.5% TCA was prepared from stock solutions. 6 mL of the working reagent and 3 mL of sample (urine or saline solutions containing added protein) were mixed together and then centrifuged at 3700 rpm for 20 min (80 mg/L Ponceau S and 3% TCA final concentration). The supernatant was removed by suction and 2 mL of 1 M NaOH was added to the precipitate and the absorbance was read at 550 nm. The benzethonium chloride method was the Roche U/CSF (Roche Diagnostics Co., Indianapolis, IN) and was analyzed on a Hitachi 917 according to the manufacturer's instructions. Dye binding assays were: Randox Total Protein Urine (Randox Laboratories, Crumlin, UK), Beckman M-TP (Beckman Instruments, Brea, CA), and Vitros 950 UPRO (Ortho Clinical Diagnostics, Rochester, NY). The Randox and Beckman methods used the pyrogallol red dye and were analyzed on a Beckman LX-20. The Ortho Clinical assay uses a pyrocatechol violet dye and was analyzed on a Vitros 950. All assays were corrected for proportional bias with a human albumin calibrator diluted in saline.

Linearity and precision

Samples were prepared in a 0.9% saline solution at concentrations of 0.427, 0.214, 0.142, 0.047, 0.016, 0.005, and 0.0018 g/L of human serum albumin. Samples were frozen at −20°C until used. Each sample was analyzed in duplicate and the measurements were repeated for 3 days. Specimens were kept at 4°C between measurements.

Accuracy and recovery

Urine samples were obtained from five normal subjects and were assayed for baseline urinary protein concentration. Then, aliquots of the 5 urine samples and a saline solution were supplemented with different proteins: albumin (284 mg/L), gamma-globulins (200 mg/L), polypeptides (200 mg/L), and mixed protein (200 mg/L). The urinary protein concentrations of these aliquots were determined by the five assays described above.

In a second set of experiments, aliquots of the same urinary samples were filtered on a 10-kDa membrane to remove any protein over 10 kDa. The efficiency of the filtration was verified by the addition of albumin to a urine sample before the filtration and the filter was shown to remove approximately 95% of the signal by the Ponceau S/TCA assay. Five original urine samples and their filtrates (<10 kDa) were tested to assess the response of the assays to a protein free urinary matrix.

Validation of methods in the normal and low protein range

Urine (10 mL) from 50 normoproteinuric and 20 low proteinuric subjects was collected and frozen until used. For the analysis, samples were thawed, centrifuged to remove crystals, aliquoted, and assayed by the five different assays.

Statistical analysis

Regression analysis was performed with a standardized principal component analysis as described by Passing and Bablok [7]. Bias plots were generated with the Ponceau S/TCA method as the reference.

Results

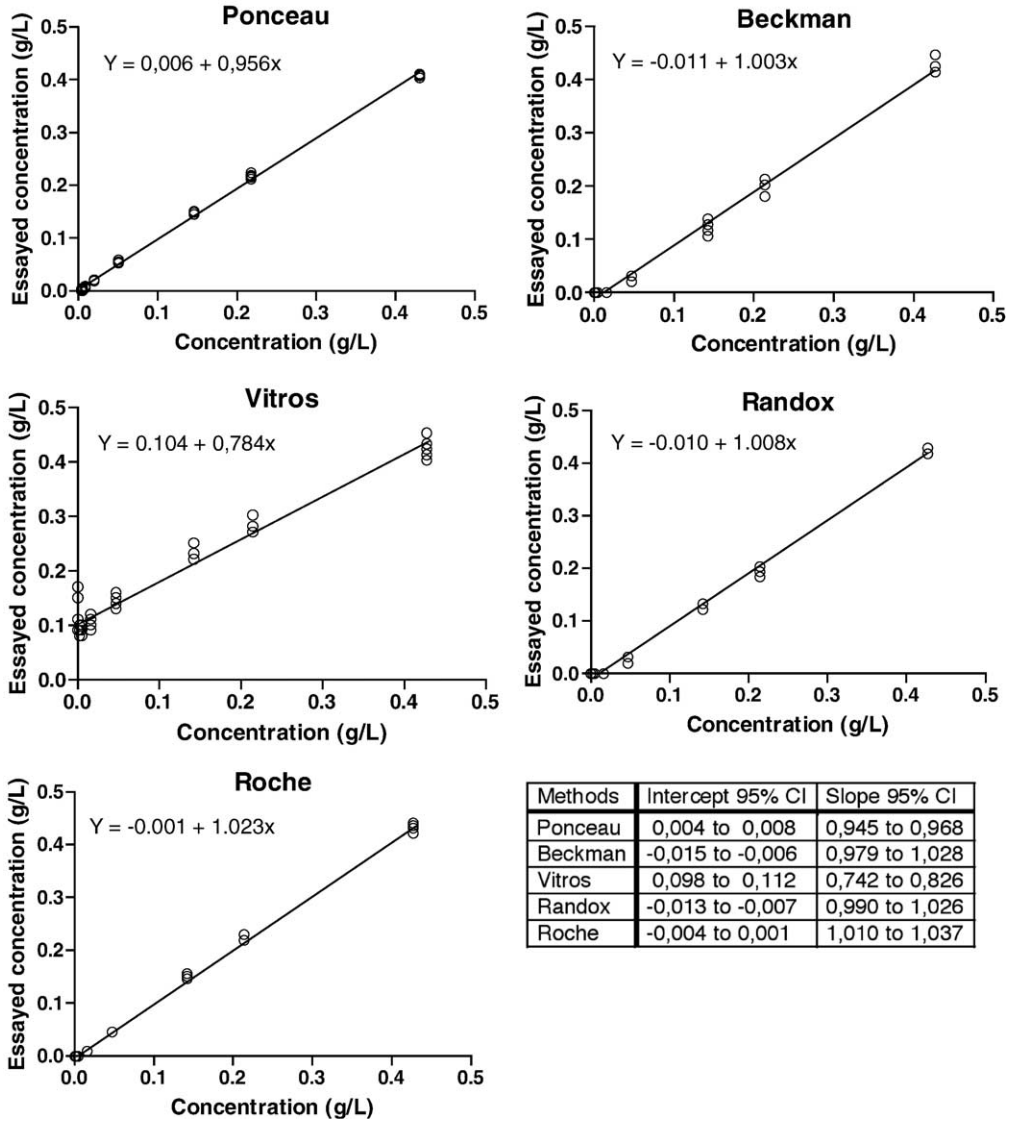
Linearity and precision

Linearity plots were obtained for all five assays (Fig. 1). All assays evaluated showed a linear relationship with the concentration of human serum albumin present in the samples. The Vitros assay showed a positive bias in a saline matrix (150 mM) when compared to the Ponceau S/TCA assay.

Fig. 2 shows the coefficients of variation in relation to the concentration for each of the five assays. Assays were used as recommended by the manufacturers without any modifications of the methods. The analytical range of the different assays varies from 0.05 to 0.06 g/L, depending on the manufacturer. Any measurement below the analytical range yielded suppressed results and precluded the exact estimation of precision for a concentration below 0.15 g/L, except for the Ponceau S/TCA assay. All assays show a CV ≤10% at a concentration of 0.15 g/L. The functional sensitivity of the Ponceau S/TCA assay at a CV of 10% is around 0.01 g/L.

Accuracy and recovery

Accuracy was estimated by using the Ponceau S/TCA as the reference method (Fig. 3). All automated assays



Methods	Intercept 95% CI	Slope 95% CI
Ponceau	0,004 to 0,008	0,945 to 0,968
Beckman	-0,015 to -0,006	0,979 to 1,028
Vitros	0,098 to 0,112	0,742 to 0,826
Randox	-0,013 to -0,007	0,990 to 1,026
Roche	-0,004 to 0,001	1,010 to 1,037

Fig. 1. Linearity plots with the equation obtained for each of the five assays evaluated. The table reports the 95% confidence intervals of the intercepts and the slopes for all assays.

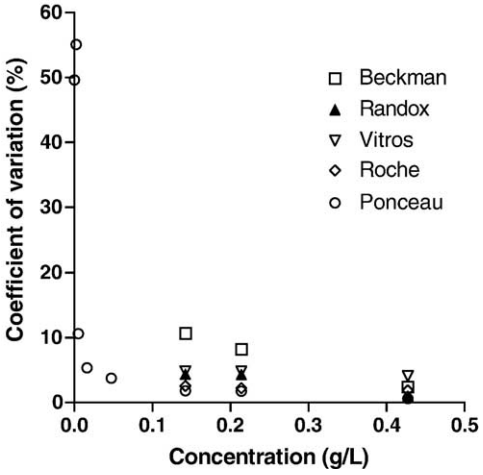


Fig. 2. Coefficient of variation profile in relation to total urinary protein concentration for each assay evaluated.

showed a small negative bias for total urinary protein levels over 0.1 g/L but below this level, an important positive bias was observed with varying intensity for each assay. Correlations between assays are shown in Fig. 4. Again, for values below 0.1 g/L, poor concordance was established for all assays.

Recovery studies were performed with the addition of human serum albumin, gamma-globulins, polypeptides (MW from 0 to 10 kDa), mixed proteins, and Tamm–Horsfall protein to a saline solution and patient urine samples (Fig. 5). All assays showed higher recovery with albumin and lower recovery with gamma-globulins. The Beckman and the Randox assays recovered 51.4% and 16.7%, respectively, of the added polypeptides whereas the other assays had recoveries below 10%. The recovery of the protein mixture was between 56.1% and 76.0%, depending on the assay. Tamm–Horsfall protein was recovered below 10% for all assays.

Effect of ultrafiltration

Removal of protein by ultrafiltration with a 10-kDa filter yielded surprising results. The Ponceau S/TCA, Roche and Randox assays showed low residual signal in the protein-free filtrate. However, the Beckman and Vitros assays generated a signal between 50% and 60% of the pre-filtration level after protein removal (Fig. 6).

Discussion

Most clinical laboratories now use automated methods to measure urinary proteins but these methods were not thoroughly evaluated for normal or slightly abnormal

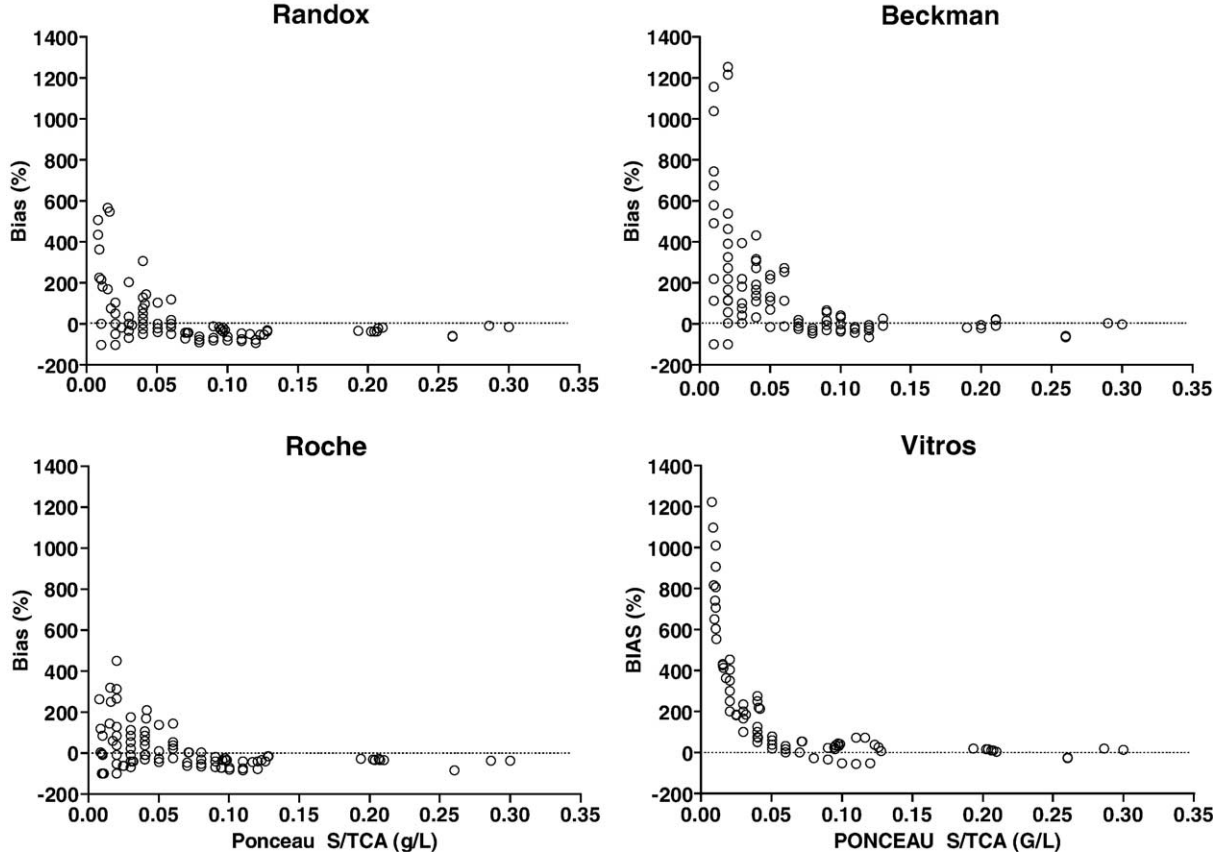


Fig. 3. Bias plots for each assay compared to the reference method (Ponceau S/TCA).

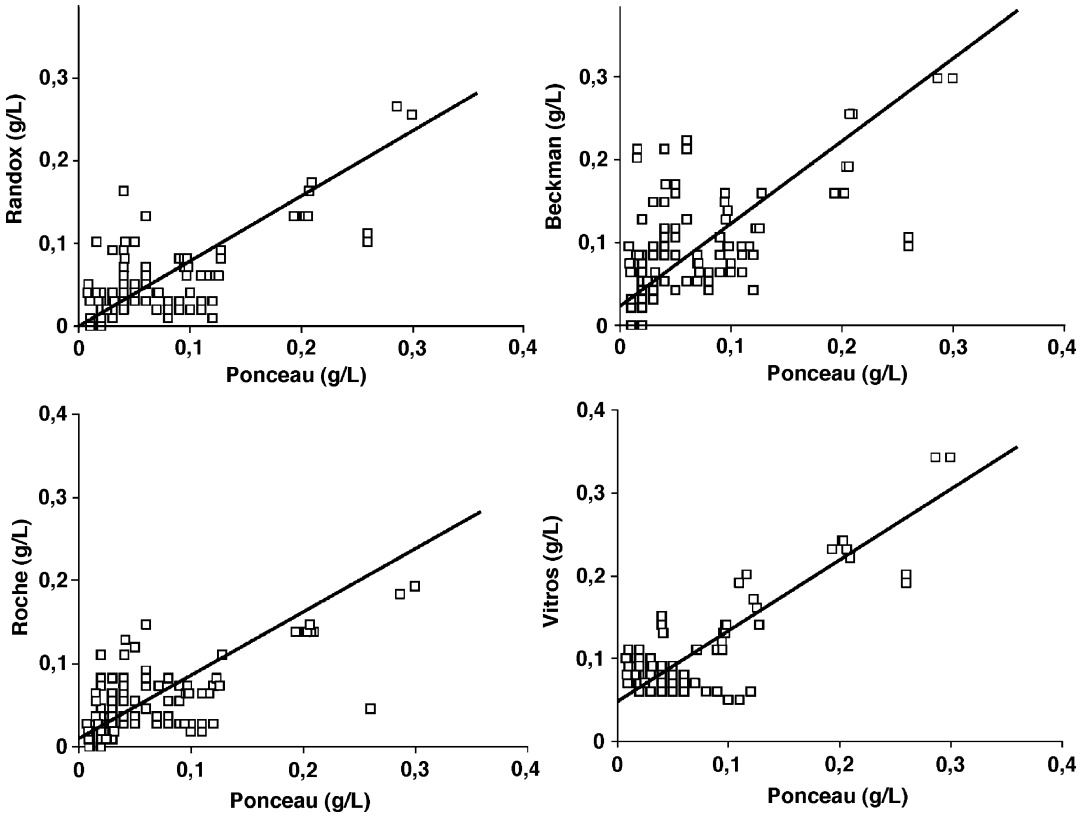


Fig. 4. Correlation plots between dye binding assays and the reference method (Ponceau S/TCA). Curve fitting was obtained by standardized principal component analysis as described by the Passing method [7].

proteinuria. We tested the performance of four automated assays often used in clinical practice for the measurement of urinary protein in or near the normal range. Our study shows that the manual Ponceau S/TCA assay performs better than the automated assays, particularly at lower concentrations. The functional sensitivity of the Ponceau S/TCA assay at a CV of 10% is around 0.01 g/L. It is, therefore, the most sensitive method used in this study. Although linearity does not seem to be a problem for the methods evaluated, poor precision and high bias were found with all automated assays.

The Roche assay is a protein agglutination assay while the other three are dye binding methods. They all show good linearity and acceptable recoveries of a large protein like albumin (Figs. 1, 5). Tamm–Horsfall (TH) was not significantly detected by the automated assays and the Ponceau S/TCA assay. The mucin-like characteristics of TH explain its resistance to dye binding and precipitation with many protein precipitants. However, the role of Tamm–Horsfall in human diseases is not substantiated [8–10] as it contributes little to the measured proteinuria even in traditional manual assays. Tamm–Horsfall recovery assays showed that none of the assays react sufficiently with the glycoprotein to explain the variations of urinary protein measurements seen in our study.

In Fig. 1, the Vitros assay shows a significant *y* intercept (0.1 g/L). This intercept is close to the upper limit of normal, expressed in g/L. It is already acknowledged that dilution of urine in water or saline solutions does not yield the same result in the Vitros assay [11], supporting the fact that this dye binding assay is influenced by ionic composition.

The average normal proteinuria is probably below 75 mg/L or 0.075 g/L [1–4]. This level represents approximately the stated analytical sensitivities of the automated assay (Fig. 2). All automated methods use only a few microliters of urine as opposed to the manual method that concentrates the protein from 3 mL of urine. We think this is an important factor contributing to the low sensitivity at a CV of 10% for the Ponceau S/TCA assay.

Our results show very large biases (Fig. 3) and poor correlation (Fig. 4) with the Ponceau S/TCA assay, especially in the normal proteinuria range (<0.15 g/L) where correlation is essentially absent. Many factors could have contributed to this lack of concordance. First, imprecision of the assays might obscure any relationship between methods. The fact that a good linearity was observed for all assays would argue against imprecision as a significant contributor although these experiments were done in a normal saline matrix with purified albumin. Since these linearity experiments were not performed in urine, we cannot assume that the urinary matrix does not induce imprecision and hence contribute to the lack of correlation seen in Fig. 4.

In our view, interference is probably the most important contributor to the differences between methods and to the

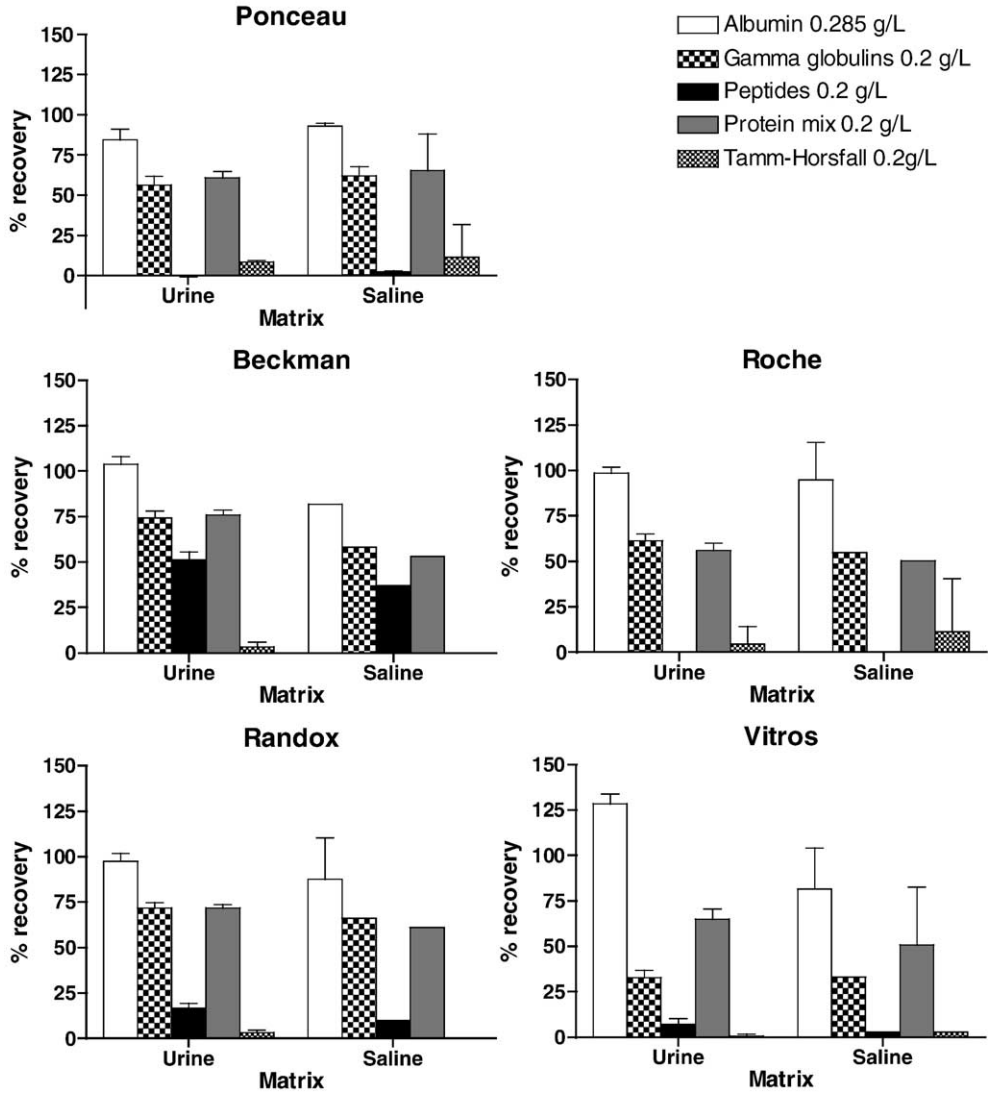


Fig. 5. Recovery studies of human serum albumin (0.28 g/L), gamma-globulins (0.2 g/L), polypeptides (0.2 g/L), protein mix (0.2 g/L), and Tamm–Horsfall protein (0.2 g/L) in saline and 5 urine samples. Results shown are averages ± CI (95%) obtained from duplicate measurements.

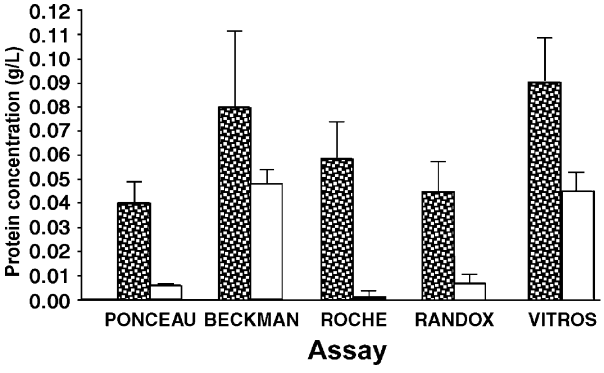


Fig. 6. Protein concentration of unfiltered normal urine samples (filled bars) and of the same urine samples filtered on a 10-kDa membrane (unfilled bars). Results shown are averages ± CI (95%) obtained from duplicate measurements of 5 urine samples before and after ultrafiltration.

positive mean bias observed for protein determinations below 0.1 g/L (Fig. 3). Urine is a complex mixture with large variations in osmolality, ionic composition, and metabolite content. As stated in the company package insert, the Vitros method is sensitive to sodium chloride concentrations above 150 mM. Various metabolites normally found in urine in different concentrations might compete for binding with the dye while other urinary constituents may induce a variable degree of color change with the reagents.

The presence of a complex and variable mixture of proteins (albumin, globulin, Tamm–Horsfall, polypeptides) reacting differently with the different protein reagents may be another explanation [12–14]. Small peptides produced a very significant signal with the Beckman and the Randox assays but did not interfere with the Roche and the Ponceau S/TCA assays. In light of this, part of the bias and imprecision at low values could be explained by a differ-

ential response to polypeptides with some assays not reacting while others reacted with higher intensity. Moreover, interferences cannot be attributed to a specific drug in our samples as the effects would be limited to only a few samples, which was not the case. Our results are consistent with metabolite or small peptide interference because of the reaction of certain assays with polypeptides (Fig. 5) and with <10 kDa filtered urine (Fig. 6).

The calibration material can also produce varying responses between methods depending on the protein source (animal versus human) [15]. The use of a common calibrator can alleviate this problem and improve the interassay comparisons [16,17]. Different calibrator strategies have been investigated using albumin or albumin/globulin mixtures [6,18,19]. In accordance with our results, some authors have suggested the abolition of urinary total protein measurements because of the lack of reliability of the assays [20]. A less drastic approach would be to use a manual measurement of total urinary protein for patients not known to have a proteinuria over 0.5 g/L.

Our results strongly suggest that the reference range for urinary protein will vary depending on the methodology used. Reported reference values for urinary protein output differ widely probably due to the sources of variation explained above [1–4]. Generally, proteinuria below 0.15 g/day is considered normal in an adult and translates to 0.1 g/L with a urinary daily volume of 1.5 L. This study demonstrates that the automated assays tested are inadequate for the measurement of urinary protein in the normal range.

In conclusion, the Ponceau S/TCA assay offers a precise and accurate manual alternative for the measurement of urinary total proteins. Although the automated assays are probably adequate for measurement of urinary proteins above 0.5 g/L, they are not suitable for normal proteinuria. Laboratories should inform clinicians about the limitations of these assays so that care can be taken before initiating costly investigations or treatments.

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