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Original Article

A novel semi-automated method for urine protein assay associated with calculation of sigma metric

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ABSTRACT

Introduction: a novel semi-automated method for urine protein assay was introduced, validated and also analytical performance evaluation was conducted for the clinical laboratories practice. **Materials and method:** Introduced method was calibrated based on multipoint calibration with human based biochemistry calibrator. All of the method validation procedure was done according to the CLSI guidance. Finally, total error, sigma metric and performance of quality was evaluated. **Results:** Results indicate the good correlation with Biuret and pyrogallol-red methods in ranging from 0.33 to 6760 mg/dl. Method was linear up to 6760 mg/dl and was highly sensitive compared to pyrogallol-red method. Based on sigma scale, introduced method have good performance in broad measuring range and based on comparison practice, sigma was >6 in all decision levels for urine protein concentration. **Conclusion:** In conclusion, novel semi-automated method can be selected as preferred method in reference laboratory. Also, IVD manufacturers can use sigma scale results to improve diagnostic products.

Keywords: Urine protein, Quality control, CLSI, Total error, Sigma metric, IVD

INTRODUCTION

Proteinuria is a risk factor for the development of End Stage Renal Disease (ESRD) in the general population and powerful predictor of renal outcome in Chorionic Kidney Disease (CKD) (1). Positive urine protein results can be transient or insignificant laboratory finding, although proteinuria can be results from both renal and nonrenal causes. Proteinuria can be classified as glomerular (mainly albumin), tubular (low molecular mass protein), overload/overflow

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(mainly bence jones protein) and post-renal proteinuria. Urinary total protein measurement continues to have a place in the repertoire of clinical laboratory test, particularly in the clinical assessment of patient with renal disease (2, 3).

In clinical laboratory, total protein measurement is more difficult in urine than in serum sample. The concentration of urine protein is normally lower than serum and it is common for there to be large sample-to- sample variation in the amount, pH, and composition of protein. In addition, effect of interfering nonprotein substance is highly relative to the protein concentration and very variable, and the inorganic ion content is high (4).

All these factors affect the Total Analytical Error (TAE) of various urine protein methods (5). In routine clinical practice, total urine protein measurement is automated using either a turbidimetric or a dye-binding method. However automated assays show poor accuracy and high imprecision compared to chemical methods (6). In addition to the methodological differences between the various automated urine protein assays, calibration has been found to be one of the major determinants of method comparability, as it has been reported by United Kingdom National External Quality Assessment Scheme (UK NEQAS) and College of American Pathologists (CAP) (7). Effect of poor accuracy and high imprecision was illustrated by sigma metric in In Vitro Diagnostic (IVD) manufacturers and clinical laboratories (8).

Sigma metric is a well-known quality management approach that uses multiple tools to achieve the goal of reducing errors and defect in any process. In clinical laboratory, sigma metric uses to measure quality in an objective and quantitative manner combining three traditional elements to evaluate assay performance: the total allowable error (TEa), bias and precision. A higher sigma metric value means fewer analytical errors and high quality and test results are acceptable, and low sigma value means low quality and test results are unacceptable (9). The aim of this study was to introduce a novel semi-automated method for urine protein assay with a broad measuring range associated with reducing imprecision and inaccuracy. We also aim to perform method validation based on Clinical and Laboratory Standards Institute (CLSI) guidelines, finally sigma metric is calculated.

MATERIAL AND METHODS

Equipment: Measurements were performed using Mindray (Mindray Bio-Medical Electronic, China) model bs-380. We verified that instrument pipette and spectrophotometer CVs were <0.05% weekly, by using a solution of 0.18 gr/dl and 0.36 gr/dl potassium dichromate in 0.005 mol/l H2SO4.

Reagent, Controls and calibrator:

Blank Reagent: HCl solution was prepared by dissolving HCl (CAS No: 7647-01-0, Arman Sina, chemical and Pharmaceutical Co., Iran) in deionized water to final concentration of 1.25%. The HCl blank solution was used to reduce pH dependent color interference from urinary chromogens.

Working Reagent: TCA solution was prepared by dissolving TCA (CAS No: 76-03-9, Lot No: K4645110 233, Merck, Germany) in deionized water to final concentration of 1.5%. Then, NaCl (CAS No: 7647-14-5, Lot No: L319120500-0714-0111, Biochem chemopharma, France) was added to final working reagent. All solutions are stable for several months at room temperature. The optimal confirmation condition of reagent was obtained by data analysis, using Design of Experiment (DOE) software version 11. The amount of NaCl, TCA, HCl and optical density (OD) were selected as effective factors.

Controls and Calibrators: Human based biochemistry calibrator traceable to NIST 927d reference material for total protein (Cat No: 5-175, Lot No: 902-01A, EXP. Data: 11-2020, Cormay Co. Poland), Human based biochemistry control material (Cat No: 5-172, Lot No: 812- 07D, EXP. Data: 11-2020, Cormay Co. Poland).

Patient samples: Study was performed at 9th Dey Manzariyeh Hospital, Isfahan, Iran. Of the entered samples, 180 urine samples, 12 blood samples, 22 CSF and 14 synovial samples were selected with covering a wide range of protein concentration. All samples were collected without preservative, centrifuged (2500g for 10 min) and stored at -70 ^{0}C immediately before protein assay, the samples were thawed at room temperature.

Standardization and assay procedure: No international reference material is currently available for urine protein, which is in agreement with international federation of clinical chemistry (IFCC). Therefore, we used Cormay human based biochemistry calibrator with 6760 mg/dL value to multipoint calibration. The Mindray bs-380 sample tray was loaded with controls and specimens, consisting of water Blank, 16 calibrators in ascending order of concentration. Each samples, controls and calibrators were reacted with TCA and HCl in different cuvette but in same condition. All absorbance reading was transmitted to external software based on excel which constructed a calibration curve by multipoint curve fitting and calculated the results of samples and controls. To fitting the calibration curve, 16 points were employed with dilution of calibrator to cover a

wide range from zero to 6760 mg/dL. Control Seronorm (Seronorm Human, Cat No: 200805, Lot No: 1512606, EXP. Data: 2-2020, SERO CO. Norway)

The auto-analyzer was programmed as follow:

Description	**Item 1**	**Item 2**	**Item 3**
Item Name	HCl	TCA	Final
Unit	Abs	Abs	Calculation Item: TCA-HCl
Reaction. Type	Endpoint	Endpoint	
Primary. Wavelength	660 nm	660 nm	
Sample volume	40	40	
R1 volume	200	200	
Reagent blank	9/10	9/10	
Reaction time	24/25	24/25	
Direction	Increase	Increase	
Calibration Type	Linear 1point	Linear 1point	
Replication	3	3	

Imprecision: The imprecision study was performed according to the CLSI EP-5A3 guideline using eight controls (direct and diluted control as #1 (1:2); #2 (1:10); #3 (1:50); #4 (1:100); #5 (1:200); #6 (1:400) #7 (1:800)) at protein concentration ranging from 8.37 to 6700 mg/dL (10). Imprecision study was done by using Cormay human based biochemistry control material with 6700 mg/dl target value for total protein. Control seronorm human was used as third-party control with 7000 mg/dL target value for total protein.
Controls were randomized and measured in duplicate with two runs per day for total of 20 days. The Standard Deviation (SD) and Coefficient of Variation (CV) were calculated for repeatability (within- run precision), intermediate imprecision (between-run precision) and total imprecision.

Bias: According to the CLSI EP15-a3 guideline (11), practical determination of bias relies on comparison of the men of the results ($\bar{X}$) from the candidate method with a suitable reference value. Three general approaches are available:

1. Analysis of reference materials (RMs).
2. Recovery experiment using spiked samples.
3. Comparison with results obtained with another's methods.

Determination of bias was carried out with followed approaches:

a. Practicing in Randox International Quality Assessment Scheme (RIQAS, cycle 56 sample 4) as external quality control was conducted based on consensus concentration in modified control samples. To determine the bias from RIQAS, the sample was measured triplicate in one run. Results were compared with the results obtained from turbidimetry, pyrogallol red, biuret reaction-direct, vitros, biuret reaction with ppt methods and mean of all methods peer group (12).
b. Comparing serial dilution of spiked natural sample in the calibration curve was performed (13). The serum sample with a 6000 mg/dL concentration for total protein was used in nine serial dilutions as follow: #1 (1:2); #2 (1:4); #3 (1:8); #4 (1:16); #5 (1:32); #6 (1:64); #7 (1:128); #8 (1:256) and #9 (1:512). All of them measured in triplicate with one run. Finally, observed results were compared with expected results. Results analysis was performed using passing bablok analysis.
c. Comparison method was performed on 60 normal urine samples, 12 blood samples, 22 CSF and 14 synovial samples and results analysis was performed using passing bablok regression (14). In order to comparing method, biuret and pyrogallol red dye-binding assays kits from Cormay Company were used. Biuret method was used to assaying serum and synovial samples and pyrogallol red dye-binding method was used to assaying normal urine sample. Assay, calibration and quality control for maintained kits performed exactly as recommended by manufactures. Biuret and pyrogallol red kits were calibrated weekly and reagents and calibrators from a single lot were used. We verified used lot to confirm to the manufacture analytical range by recovery test.
d. In the absence of suitable RMs, recovery studies were used to give an indication of the likely level of bias (15). to perform recovery test, both low and high concentration samples was needed. To achieve best result in recovery test, we used serum sample with 6000 mg/dl and urine sample with 3200 mg/dl concentration for total protein as high concentration samples and urine samples with 60 and 20 mg/dL concentration for total protein as low concentration sample. Finally, recovery percent calculated according to the following protocol:

i. Total protein concentration of both serum and urine sample measured in triplicate.
ii. 100 µl of serum sample was added to 900 µl of urine sample and mixed.
iii. Total protein concentration of above mixed sample was measured in triplicate.
iv. Recovery was calculated according to following formula: A: (100 µl/ 100 µl + 900 µl) * high concentration sample (mg/dl) B: (mixed sample concentration – urine sample concentration) Recovery (%): (B/A) *100
v. Bias (%): recovery (%)-100

Detection Limit: The urine protein detection limit was determined according to CLSI EP17-A2 guideline (16). For the Limit of Blank (LoB) determination, deionized water as zero standard was run over six days with four runs per day and five replicates per run. The 95th percentile of the upper reference limit was calculated from a total 120 replicates.

Linearity: Linearity of the dynamic range was evaluated according to CLSI EP06-A guidelines (17). Normal serum sample with 7000 mg/dL value and low urine sample (<1mg/dL) were used at neat and mixed samples to make nine evenly distributed sample concentrations. Measured protein value was plotted against the expected protein concentration and linearity was determined using the polynomial regression method.

Total Analytical Error, Sigma Scale and Quality Goal Index: Total Analytical Error (TAE) was performed according to the westgard recommendation protocol (18). To determine the TAE according to westgard recommendation (%TAE: %bias + 1.65*CV %), obtained results from bias and imprecision in several concentrations were used. The TAE was reported as percent difference and compared to the total analytical error goal. Allowable Total Error (TEa) is the best expressed tolerance limit in clinical laboratory that combine both imprecision and bias of a method to calculate the impact on a test result. The Ricos biological variability (desirable target values, in contrast to the minimal or optimal target value) and Royal College of pathologists of Australasia (RCPA) data bases were used as reference for TEa specification. Both mentioned sources of TEa were used to calculation sigma metric. The Sigma metrics was calculated based on mean of bias obtained from all of the control's levels and recovery level. Sigma metrics was calculated using standard equation:
Sigma metric: (TEa - Bias) / CV [all value expressed as percent (%)].

The Quality Goal Index (QGI) was calculated using the formula: QGI = Bias / (1.5*CV). This index can help determine the main reason why the testing performance of a urine protein assay project yields a lower sigma level. A sigma value less than 4 was used as the benchmark for the QGI analysis of the urine protein assay in this study. QGI less than 0.8 indicates that the precision of measuring protein at the relevant concentration needs to be improved, whereas a value greater than 1.2 indicates that the accuracy of measuring protein at the relevant concentration needs to be improved. A QGI value between 0.8 and 1.2 indicates that accuracy and precision needed to be simultaneously improved (19).

Statistical analysis: The SPSS version 18.0 and Excel software were used to perform a descriptive statistical analysis.

RESULTS

Calibration curve: The calibration curve studied extended from zero to 6760 mg of protein per deciliter. The calibration curve was slightly sigmoidal. It was linear between zero and 1352 mg/dl (Fig. 1, Table 1). We have used 16 concentration of calibrator in order to cover a wide range.

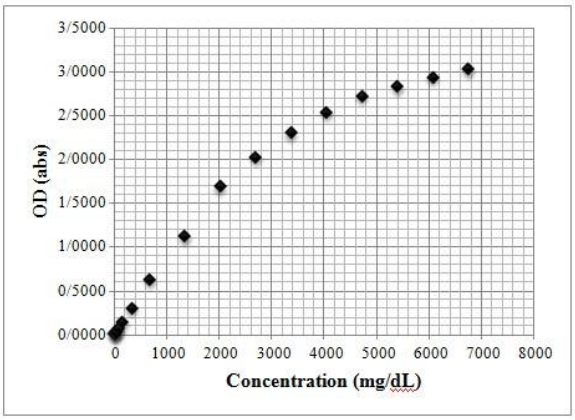

Figure 1: A typical calibration curve for the semi-automated urine protein method.
Each point represents the absorbance range and mean of three replicates.

Table1. The results obtained from mean and CV of 17 calibration points for TCA and HCl.

Calibrator No.	Concentration (mg/dL)	TCA			HCl			Final OD (TCA-HCL)
		Mean	SD	CV%	Mean	SD	CV%	
1	**0**	0.0001	0.0000	0.00	0.0000	0.0000	0	0.0001
2	**10.5**	0.0054	0.0001	0.93	0.0004	0.0000	0	0.0050
3	**21.12**	0.0124	0.0002	1.45	0.0010	0.0000	0	0.0114
4	**42.1**	0.0305	0.0003	0.95	0.0021	0.0000	0	0.0284
5	**84.5**	0.0717	0.0007	1.02	0.0041	0.0000	0	0.0677
6	**166**	0.1540	0.0004	0.25	0.0074	0.0001	1.35	0.1466
7	**338**	0.3131	0.0038	1.20	0.0132	0.0002	1.51	0.2999
8	**676**	0.6573	0.0015	0.23	0.0251	0.0001	0.39	0.6322
9	**1352**	1.1652	0.0239	2.05	0.0425	0.0000	0	1.1227
10	**2028**	1.7608	0.0136	0.77	0.0666	0.0004	0.6	1.6942
11	**2704**	2.1124	0.0399	1.89	0.0820	0.0009	1.09	2.0304
12	**3380**	2.4073	0.0156	0.65	0.1026	0.0008	0.77	2.3047
13	**4056**	2.6550	0.0340	1.28	0.1162	0.0013	1.11	2.5389
14	**4732**	2.8720	0.0263	0.92	0.1425	0.0012	0.84	2.7295
15	**5408**	2.9848	0.0053	0.18	0.1564	0.0009	0.57	2.8284
16	**6076**	3.1189	0.0262	0.84	0.1807	0.0007	0.38	2.9381
17	**6760**	3.2231	0.0173	0.54	0.1905	0.0020	1.05	3.0326

Imprecision: Repeatability, intermediate and total imprecision of the method were determined by 20 replicate analysis of eight controls samples with concentration ranging from 8.37 to 6700 mg/dl. Results of imprecision study are summarized in table 2.

Table2. Repeatability, intermediate and total imprecision of the urine protein assay. SD and CV were calculated based on measurement of each control according to CLSI EP-5A2 guideline.

Control	Mean	Repeatability		Intermediate		Total imprecision	
	n=20	SD	CV%	SD	CV%	SD	CV%
mg/dL	mg/dL	mg/dL		mg/dL		mg/dL	
8.45	8.35	0.3	3.6	0.3	4.1	0.4	4.7
16.9	17.1	0.4	2.3	0.6	3.8	0.7	4.1
33.8	34.2	0.8	2.5	1.1	3.3	1.5	4.3
67.6	66.9	1.4	2.1	1.9	2.9	2.1	3.1
135.2	134.2	2.2	1.7	2.9	2.2	3.5	2.6
676	679	7.4	1.1	12.9	1.9	14	2
3380	3420	27	0.8	37	1.1	41	1.1
6760	6720	47	0.7	67	1.0	70	1

Bias

Calculated bias from EQA was provided by RIQAS program. The results of EQA study are summarized in table 3. As the table show, results of semi-automated urine protein method compared with turbidimetry, pyrogallol red, biuret reaction-direct, vitros, biuret reaction with ppt methods and mean of all methods peer group obtained from RIQAS program with the following formula: Bias (%): (measurement value – target value) / target value * 100. The mean of the EQA (RIQAS) results reported by clinical laboratories that used the different type of method and instrument was used as the target value for urine protein analyte (Table 3).

Table 3: Comparison the results of Semi-automated urine protein method with mentioned methods in RIQAS program.

CYCLE 5,5 SAMPLE 12, 02/12/2019			
	N	Mean	Bias of Semi-automated method (%) *
All Methods	680	12.856	1.2
Turbidimetry	353	11.69	11.3
Pyrogallol Red	236	14.828	-12.3
Biuret reaction - direct	75	13.248	-1.8
Vitros	15	19.18	-32.0
Biuret reaction with ppt	4	13.94	-6.7
Semi-automated method		13.01	

*: The bias of the semi-automated method has been calculated by comparing the results with the methods mentioned in the program as separately.

Comparison result of serum sample serial dilution with calibration curve was show in table 4 and fig. 2. All of the dilution points were compared, and bias was calculated for each point. Expected value of serum protein concentration was determined with biuret method. The higher correlation was determined between semi-automated result and expected value r: 0.9996)

Table 4: Summarized results of bias calculated with comparing the observed results and expected value for serial dilution of spiked natural sample.

Expected (mg/dl)	Observed mg/dL)	Bias %
23.4	23.9	2.1
46.8	47.1	0.6
93.7	94.2	0.5
187.5	187	-0.3
375	377	0.5
750	753	0.4
1500	1490	-0.7
3000	3100	3.3
6000	5950	-0.8

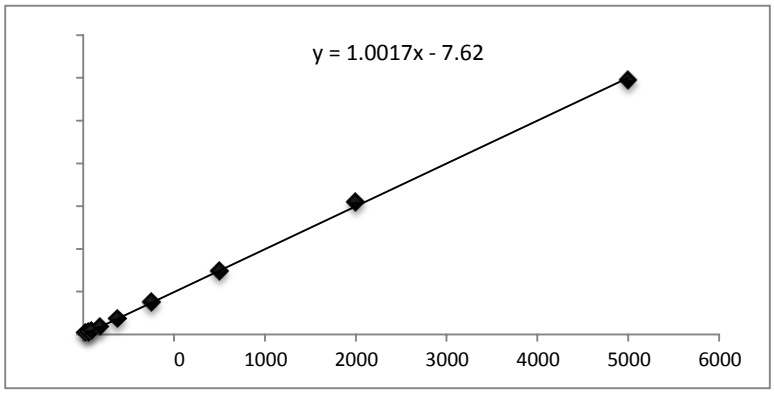

Figure 2: Comparison results of serum sample serial dilution with expected value.
The line of the identity is given as the dashed line, the passing bablok regression line y = 1.0017 x – 7.62 as the solid line, the spearman's correlation coefficient r being 0.9996.

The patient urine, serum, CSF and synovial samples used for the comparison study ranged from 1 to 6670 mg/dl. The higher correlation was determined between semi- automated urine protein method and biuret (for serum and synovial samples) and pyrogallol red (for normal urine and CSF samples) methods. Passing bablok regression analysis gave r: 0.9999 slope 0.1.002, intercept of 0.99 mg/dl and average bias is -6.2%. Correlation data show in fig 3.

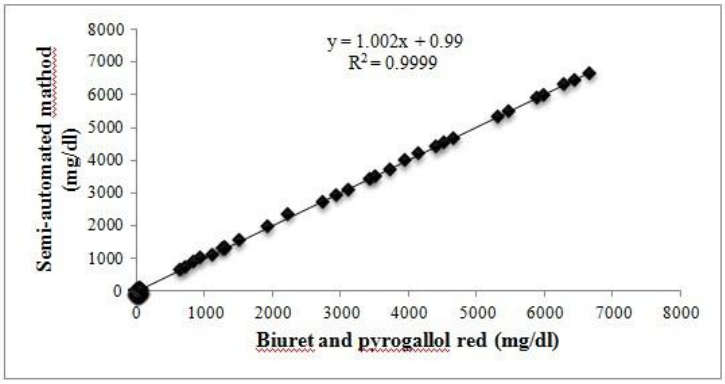

Fig3. Method comparison between the semi-automated urine protein and biuret and pyrogallol red method analyzed via passing bablok regression. Protein values were measured for 108 samples across the range of the

assay (1 – 6670 mg/dl). The graph shows an example of the result obtained by semi-automated urine protein and biuret and pyrogallol red method. The line of the identity is given as the dashed line, the passing bablok regression line y = 1.002 x + 0.99 as the solid line, the spearman's correlation coefficient r being 0.9999, also Constant systematic error: 2.58 mg/dl, proportional systematic error: -0.17% and average bias is -6.2%.

The recovery of the assay was assessed by adding sample with 6000 and 3200 mg/dl concentration to 60 and 20 mg/dl concentration for total protein respectively and the analytical recovery was 98% and 96.8% respectively. Therefore, we can conclude that there is no matrix effect that influences the results (Table 5).

Table5. Summarized results of recovery test

	Low sample	High sample	A † (mg/dL)	B‡ (mg/dl)	R (%) *	Bias%
sample 1	60	6000	600	588	98	-2.0
Sample 2	20	3200	320	310	96.8	-3.2

*: Recovery: (B/A) *100 †: added concentration value ‡: mixed sample concentration – urine sample concentration

Detection Limit: The LOB and LOD ranged from 0.018 mg/dl to 0.0712 mg/dl for mindray BS-380 and the LOQ was 0.338 mg/dl. The LOQ were determined as the minimum concentrations at which TAE were below 40% according to the Ricos goal data base (Fig. 4). If RCPA limitation was used, the LOQ is 2.5 mg/dl. Based on RCPA, TEa for the urine protein 24hr is 10%.

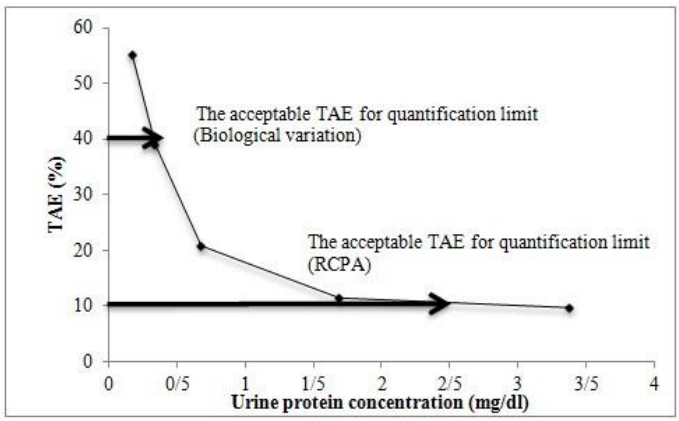

Figure 4: Limit of quantification for urine protein semi-automated method.

Linearity: Fig. 5 shows the results of linearity for the semi-automated urine protein method. The linearity study was conducted based on CLSI EP06-A guidelines using polynomial regression method. The semi-automated urine protein presented excellent linearity in the measuring range (0.33 – 6760 mg/dl). Expected value of serum protein concentration was determined with biuret method

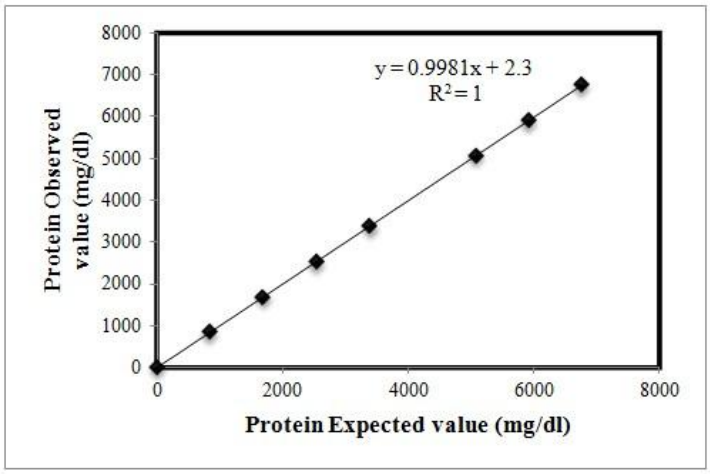

Figure 5: Linearity experiment for the semi-automated urine protein assay according to CLSI EP06-A guidelines. A high sample and low sample were analyzed in addition to 9 evenly distributed dilutions which

were created by mixing the high and low sample. All diluted sample were measured in duplicate. All result is calculated in mg/dl.

Total Analytical Error and Sigma metric: To calculation of TAE essential statistical consideration combining bias and imprecision were used. TAE was calculated for all of the controls levels that which bias was calculated. Results of TAE are summarized in Table 6. As the table show, all of the TAE percent is less than 10% and also maximum percent is for 34.2 mg/dl. In compared to TEa, in both of the biologic variation and RCPA sources, TAE is less than TEa.

Table 6: summarized results of TAE calculated based on combining bias and imprecision in different methods of obtaining bias.

Description	Mean (mg/dl)	CV (%)	Bias (%)	TAE (%)
IQC* using human based biochemistry control material	8.35	4.7	-1.18	6.6
	17.1	4.1	1.18	7.9
	34.2	4.3	1.18	8.3
	66.9	3.1	-1.04	4.1
	134.2	2.6	-0.74	3.6
	679	2.0	0.44	3.7
	3420	1.1	1.18	3.0
	6720	1.0	-0.59	1.1
EQA† (RIQAS) mean of all methods as target value	13.01	0.5	1.2	2.02
Comparison of spiked natural sample serial dilution with calibration curve	23.9	1.1	2.1	3.92
	47.1	1.0	0.6	2.25
	94.2	0.9	0.5	1.99
	187	0.9	-0.3	1.19
	377	0.6	0.5	1.49
	753	0.3	0.4	0.90
	1490	0.4	-0.7	-0.04
	3100	0.2	3.3	3.63
	5950	0.2	-0.8	-0.47
Recovery test	588	0.5	-2.0	-1.18
	310	0.7	-3.2	-2.05
Correlation analysis	891.6	0.9	-6.2	-4.7

To understand the performance of the urine protein in the semi-automated method, the sigma metrics of urine protein at the 21 levels were calculated. Complete sigma metrics for 20 levels concentration are show in table 7. According to the sigma metric levels, the of the semi-automated method was divided into six grades as world class ($\delta \geq 6$), excellent ($5 \leq \delta < 6$), good ($4 \leq \delta < 5$), warning ($3 \leq \delta < 4$), poor ($2 \leq \delta < 3$) and unacceptable ($\delta < 2$). As the table show, using RCPA TEa target, when bias was calculated from Internal Quality Control (IQC) data, at concentrations of 8.365, 17.1and 34.2 mg/dl; it has between 2 to 3 sigma metrics and is in poor grade. At a concentration of 66.9 mg/dl, the sigma metric is 3.6 and at concentrations of 134.2 and 679, sigma metric has been between 4 to 5. Also, when bias was calculated from correlation analysis, sigma metric was 4.2 at concentration of 981.6 mg/dl. According to the QGI calculated in sigma less than 4, the reason for the low sigma metric is due to the high imprecision and should be improved for data from IQC analysis.

Using biological variation TEa target, in generally the sigma value are very acceptable and indicate robust, high quality results about 6 up to high value.

Table 7: summarized results of sigma metric calculated based on Ricos goal and RCPA in different methods of obtaining bias.

Description	Mean (mg/dl)	CV (%)	Bias (%)	Sigma based on (B.V‡)	Sigma based on (RCPA†)	QGI*
IQC using human based biochemistry control material	8.35	4.7	-1.18	>6	2.4	0.17
	17.1	4.1	1.18	>6	2.2	0.19
	34.2	4.3	1.18	>6	2.1	0.18
	66.9	3.1	-1.04	>6	3.6	0.22
	134.2	2.6	-0.74	>6	4.1	
	679	2.0	0.44	>6	4.8	
	3420	1.1	1.18	>6	>6	
	6720	1.0	-0.59	>6	>6	
EQA (RIQAS) mean of all methods as target value	13.01	0.5	1.2	>6	>6	
Comparison of spiked natural sample serial dilution with calibration curve	23.9	1.1	2.1	>6	>6	
	47.1	1.0	0.6	>6	>6	
	94.2	0.9	0.5	>6	>6	
	187	0.9	-0.3	>6	>6	
	377	0.6	0.5	>6	>6	
	753	0.3	0.4	>6	>6	
	1490	0.4	-0.7	>6	>6	
	3100	0.2	3.3	>6	>6	
	5950	0.2	-0.8	>6	>6	
Recovery test	588	0.5	-2.0	>6	>6	
	310	0.7	-3.2	>6	>6	
Correlation analysis	891.6	0.9	-6.2	>6	4.2	

DISCUSSION

The quantitative methods of Urine Protein can be classified into three main approaches: chemical, dye-binding, and turbidimetric assay (20). In chemical method, biuret and Lowry reaction were used and the Lowry reaction is 100 times more sensitive than the unmodified biuret reaction but color, varies with amino acid composition of protein, Urate can interfere (21). For dye-binding methods, there was three main methods: Ponceau S, Coomassie Brilliant Blue (Bradford), and pyrogallol red–molybdate. Pesce and Strande, developed ponceau S method for determination of urine protein (22), Salo and Honkavaara subsequently modified the Pesce and Strande procedure (23). Meola et al also modified Ponceau S with using cellulose powder (24). Coomassie Brilliant G-250 dye-binding method proposed with Bradford for estimation of protein at low concentration (25). An alternative dye-binding method utilizing pyrogallol red-molybdate was described by Watanabe et al (26). The original pyrogallol red-molybdate showed good agreement for albumin (not for globulin) with the biuret method. Also, the addition of SDS to the reaction was found to increase sensitive for the determination of gamma globulins (27). Turbidimetric measurements of urine protein are widely used in clinical laboratories such as TCA, benzethonium chloride or ammonium chloride. Moreover, the turbidity varies appreciably with the chemical nature of the acid precipitant, the type of protein, the concentration of the acid, the temperature, and the time elapsed between addition of the acid and turbidimetric measurement. In routine clinical practice, the turbidimetric and dye-binding methods do not give equal analytical specificity and sensitivity for all proteins. Also, automated assays show high imprecision and poor accuracy. This may be of particular importance in detection of immunoglobuline light chain (28). The comparison of several protein assay methods is listed in table 8.

In this study, an analytical performance evaluation was conducted on a novel semi-automated urine protein assay for the clinical laboratories practice. The results clearly demonstrated that the introduced method is superior to current methods in accuracy and imprecision and measuring range. As such, its remarkable analytical performance makes it suitable for implementation in reference laboratories practice and comparison study as reference method. An LOD 0.0712 mg/dL showed significantly higher sensitivity compared to current

methods. The sensitivity of the benzethonium chloride turbidimetric method in alkali is comparable to that of the Lowry method, that is, approximately 10 mg/L (29). Although the originators of the benzethonium chloride method claimed that the turbidity produced was relatively insensitive to the albumin/gamma globulin ratio, subsequent evaluations showed that gamma globulin produces 11% to 31% less turbidity than albumin, depending on the total protein concentration (100 to 2400 mg/L) (30). Turbidimetric methods have been compared for sensitivity and bias with purified fractions of human serum proteins. Nishi and Elin concluded that these methods will give results of varying accuracy for a urine sample that contains proteins different from those in the standard material (31).

Of the dye-binding methods, Coomassie Brilliant Blue is the most sensitive. The protein–Coomassie Brilliant Blue dye complex has a high extinction coefficient and has four times the sensitivity of the Lowry method. However, as is true for the Lowry method, the amount of color development varies with the nature of the protein standard used and the nature of the protein in the sample (32). In one study, two commercially available modifications of the Coomassie Brilliant Blue method were compared to a biuret method, using urine samples. The correlations were good, despite the fact that both assays produced a lower absorbance response with gamma globulin than with albumin (30). There is considerable disagreement about the extent of linearity of the method with different modifications or protein standards. It seems clear that slight differences in reagent composition, standard, method, or dye lot can produce significant differences in the response of the assay. Perhaps the most important problem encountered with current commercially available method is the nonlinearity of results obtained on diluted samples. Both positive and negative errors are found which could lead to either overestimation or underestimation of protein results (34). Dye-binding methods using pyrogallol red–molybdate are now preferred to Coomassie Brilliant Blue in routine clinical practice. Although the Coomassie Brilliant Blue method is still the more sensitive assay, modifications to the pyrogallol red–molybdate with SDS and improved calibrators has resulted in a more accurate and reproducible assay (27). Dube et al says that Most automated assays show high imprecision and poor accuracy for the measurement of urinary protein in the normal range (35).

The quality of IVD device such as laboratory instrument, reagent and assays are an essential elements of total laboratory testing quality and analytical performance (36). Establishing analytical performance is a prerequisite for the IVD manufacturers and clinical laboratories, but it can be difficult to assess. Using sigma metric when establishing analytical performance requirements can help IVD manufacturers to optimized IVD assay product performance and also, can help clinical laboratory to ensure acceptable patient test results are reported and false rejection of results is minimized. Precision, Bias and TEa are the three traditional elements to evaluate sigma metric (37).

In accordance with these reports, we confirmed that the introduced method exhibited low CVs and high reproducibility as: CV≤ 3.6% for repeatability, CV≤ 4.1% for intermediate and CV≤ 4.7% for total imprecision based on 20-day experiment. Furthermore, the semi-automated method showed excellent linearity in the assay measuring range (0.338-6760 mg/dL) using the polynomial regression method in accordance with CLSI EP06-A.

Bias is more difficult than precision to realistically estimated than CV%. In this study, bias estimated from three approaches as comparison study, practicing in EQA and recovery test but we emphasize the comparison of results. We did not consider the control target value, because the control target value was not assigned by reference method analysis. Also, the controls used in this study may be not commutable, therefore the observed bias is only relative instead of absolute.
In method comparison studies of semi-automated assay and biuret/pyrogallol red assays there was a high correlation with r>0.999 for samples covering the relevant concentration range of total protein. This correlation study demonstrated a significant agreement of total protein value observed between the semi-automated method and biuret/pyrogallol red assay.

Today, one of the approaches to estimating bias is to participate in the EQA. EQA is a component of laboratory accreditation requirements and are an essential component of a laboratory's quality management system, and in many countries. The value and importance of participating in EQA for the laboratory depends on correct evaluation and interpretation of the EQA result. Key factors for interpreting EQA results are: 1) knowledge of the EQA material used, 2) the process used for target value assignment, 3) the number of replicate measurement of the EQA sample, 4) the range chosen for acceptable values around the target (acceptance limits), 5) and the impact of between lot variations in reagents used in measurement procedures. In RIQAS analysis as EQA, for each instrument, method and all methods group, the mean and standard deviation (SD) are calculated and at this point Chauvenet's Criterion is applied to

identify and exclude statistical outlying results from the calculations. The mean and SD are recalculated after the outliers have been excluded. Then, the Chauvenet's process is repeated. Finally, the 95th percentile of the new mean and SD is calculated. This is used to create a final upper and lower range. If any results fall outside this range they are also excluded. The final mean, SD and coefficient of variation (CV) are then calculated. The bias calculated from this program is relative. Because the sample may be not commutable and target value was not assigned by reference method analysis. A commutable EQA sample behaves as a native patient sample and has the same numeric relationship between measurements procedures as is observed for a panel of patient samples. A non-commutable EQA sample includes matrix related bias that occurs only in the EQA sample but not in authentic clinical patient samples and therefore, does not give meaningful information about method differences, howbeit we estimated matrix effect by recovery test and concluded that no matrix effect.

The choice of TEa is critical and has a major impact on the sigma metric and analytical performance, as clearly illustrated in calculation of LOQ and sigma metric (8, 16). Two common sources of TEa were chosen. Biological variation and RCPA, however ther was another source for TEa and a laboratory must decide which TEa goal is most appropriate for it (38). The sigma metric under biological variability are > 6. With RCPA, using exactly the same bias and precision value, the sigma values ranged from 2.2 to >6. So, depending on the TEa chosen, the same urine protein assay would be classified under biological variation as definitely of word class quality (sigma >6). In calculation of LOQ, the effect of the TEa source is also visible. As the results showed, when B.V is used, the LOQ much lower than when the RCPA was used. As hens et al have demonstrated, this suggest the biological variability TEa is too demanding for analytical performance and method validation (8).

There are several limitations with this approach, one of the most obvious being that this method is semi- automated method. This limitation can be improved by a closed system automatic analyzer such as Beckman, Roche and abbott diagnostic companies. Other limitations of this study include the inadequacy of clinical trials. In future studies, samples from patients with multiple myeloma and others malignancy should also be examined and the results of this method for free and bound light chain protein measurement compared with immunofixation methods. Obviously, according to the calibration method, no significant difference should be seen.

CONCLUSION

In conclusion, novel semi-automated method can be selected as preferred method in reference laboratory to compare accuracy of others commercial kits. Also, the use of new quality control approaches such as Sigma metric can play an effective role in the process of quality control and method validation process. IVD manufacturers can use sigma scale results to improve diagnostic products, also through the Sigma scale, clinical laboratories can establish analytical performance for homemade methods.

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Table8: Urine Total Protein Methods Summary Table

Method	**Sensitivity mg/L (LOQ)***	**Principle**	**Limitation**	**benefit**	**Reference**
Semi-automated	0.33	Immunoturbidimetry, multipoint calibration	Not in routine use	Very sensitive Broad measuring range Traceable to NIST 927d No sample interference	This study

Biuret (modified)	5 to 17	Proteins concentrated with TCA or ethanolic-HCl phosphotungstic acid and redissolved in0 biuret reagent (alkaline Cu2+); the Cu2+ reagent forms a colored complex with peptide bonds, which is measured at 540 nm.	Not in routine use Traceability not defines interferences by a range of pharmaceutical drugs	Very sensitive	21
Lowry	10	Folin reacts with peptide bonds, tyrosine, and tryptophan residues to produce a blue color monitored at 650 nm.	Infrequently used color varies with amino acid composition of protein urate can interfere Traceability not defines interferences by a range of pharmaceutical drugs	Very sensitive	21, 29
Benzethonium chloride	10	added to the sample, and the denatured protein precipitates in a fine suspension that is quantitated turbidimetrically	Gammaglobulin produces 11% to 31% less turbidity than albumin. Traceability not define	Frequently used method	29, 30, 31
Ponceau S	20	Precipitation of dye-protein complex, which is redissolved in alkali; color intensity is measured at 560 nm.	Infrequently used Traceability not define	…	22, 23
Coomassie Brilliant Blue	2.5	Dye binds to NH3 residues in proteins, with a resulting absorption at 595 nm	Overestimation of albumin Traceability not defines	Rapid Highly sensitive Frequently used method	25, 32, 33
pyrogallol red–molybdate	10	Dye binds to protein, causing an increase in absorbance at 595 nm.	Aminoglycosides interfere Traceability not defines Gammaglobulin less sensitive than albumin.	Frequently used method	27, 30

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