An improvised 10-min rapid Lowry's protein estimation for clinical samples

Ganesan Mahadevan[1], Renganathan Jagannathan[1], Girija Kannan[2]* and Joseph Raj Gnaniah[3]
1. Viral Research and Diagnostic Laboratory (VRDL), Government Thiruvarur Medical College, Thiruvarur, INDIA
2. Department of Microbiology, Government Thiruvarur Medical College, Thiruvarur, INDIA
3. Government Thiruvarur Medical College, Thiruvarur, INDIA
*vrdlgtmctvr2019@gmail.com

Abstract

Lowry's protein estimation method is widely used with and without modifications in research field. Conventionally, the alkaline-copper-tartrate reagent has been made fresh every time and the total assay time of Lowry's method is 30-45 min. The objectives of this study were to reduce the assay time (to 10 min) and to validate the practice of stored 5X Lowry reagent at different temperatures up to 30 days. This study was an analytical study. Standards with BSA ranging from 3 to 25 µg/well were used for optimization of assay procedure. Serum (n=25) and CSF (n=15) samples were taken for validation purposes of stored Lowry reagent. Concentrated 5X Lowry reagent (fresh or stored at 0 °C, -20 °C and -80 °C) was added. After 1N Folin or 2N Folin addition, the plates were read after 20 min or at different time points (0, 5, 15 and 20 min) respectively.

Comparison of R^2 value, precision of predictors (RSE), estimated proteins (Bland Altman plot), total proteins (ANOVA) in biofluids was made between experimental groups. The trendline of 1N and 2N Folin showed similar R^2, among which, a linear model was achieved in the latter. The estimated protein did not significantly vary between different time points and hence 0^{th} min was taken for further analysis. The total serum and CSF protein estimated by 5X Lowry reagent stored at 0 °C for up to 30 days were in accordance with fresh reagent. This improvised 10-min Lowry protocol for protein estimation is beneficial to both clinical and research laboratories.

Keywords: Lowry's, Protein estimation, Folin, BSA, CSF, Rapid.

Introduction

Since the 1950s, the Lowry method of protein estimation has been widely used in various applications[5]. Many modified protocols were published over years to make this a rapid, suitable for microgram estimate, compatible with various interfering agents[3,6,7]. The inconvenience of this method is its long assay time (usually 30-45 min) particularly when large number of samples are to be assayed. Besides, the regression analysis between optical reading and protein content does not fit in linear parameters. The copper sulphate reagent (Lowry reagent) has to be prepared fresh on the day of use. The preparation of 5X reagent used in this study is based in part upon modifications of Fryer et al's method[2]. The stability of 5X Lowry reagent was tested at different temperatures and time periods.

Material and Methods

Chemicals and Instruments: Cell culture grade 10X phosphate buffered saline (PBS), bovine serum albumin (BSA) and Ovalbumin were purchased from HiMedia (Mumbai, India). Copper sulphate ($CuSO_4$), sodium potassium tartrate (Na-K-tartrate), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH) of analytical grade were purchased from Loba Chemie (Mumbai, India). Folin-Ciocalteu phenol reagent (2N) was purchased from Loba Chemie. Micropipettes (F1 generation) and microplate FC spectrophotometer were purchased from Thermo scientific™ (Massachusetts, U.S). Stock BSA and ovalbumin were prepared in Milli-Q water at 10 mg/ml, from that, a working solution of 0.25 mg/ml was diluted with MQ water.

Aliquots of both stocks were stored at -20 °C until use. The alkaline copper-tartrate (ACT) reagent was five times concentrated as earlier method[2]. Briefly, three solutions: 0.5% $CuSO_4$ in water, 10% Na-K-tartrate in water and 10% Na_2CO_3 in 0.5 N NaOH were mixed fresh daily at a ratio of 1:1:100 (0.25 ml: 0.25 ml: 25 ml). The Folin reagent (2N) with 1:1 dilution in water and without dilution was used.

Assay procedure: Cell culture grade, flat bottom, microtitre polystyrene plate (Thermo scientific™) was labelled as per standard at different concentrations. Standards were prepared by different concentrations (3, 5, 10, 15, 20 and 25 µg per well) of BSA and the total volume was made up to 100 µl with MQ water. To all wells, 25 µl of freshly prepared ACT (FR) was added and mixed for 10 min at room temperature by shaking in Elisa microplate washer (Erba Lisa Wash 4000, Lombardy, Italy) at 1mm X (10x) speed. Finally, diluted Folin (1:1) was added to the respective wells. The absorbance was read at 620 nm after 20 min incubation in dark at RT. All assays were performed in triplicate to calculate and to compare % CV within and between plates. The remaining 5X solution was aliquoted as one ml in 1.8-ml cryotubes and kept at different temperatures: 0 °C, -20 °C and -80 °C (Remi, Quebec, Canada) for 2-, 4-, 15- and 30-days interval.

Improved 10-min protocol: Standard BSA and ovalbumin at different dilutions were taken as aforementioned experimental setup in separate plate. Instead of diluted 1N

Folin, 10 µl of 2N (undiluted) Folin reagent was added to all the wells. The well contents were mixed by repeated pipetting using multi-channel pipette until the bubbles were removed. The plate was read at 620 nm - 0^{th}, 5^{th}, 15^{th} and 20^{th} min in Multiskan™ FC microplate photometer (Thermo scientific).

Stability of 5X Lowry reagent: All the stored 5X reagents were thawed at room temperature (RT at 20 °C) after 2-, 4-, 15- and 30- days. The contents were vortexed and 25 µl of fresh or stored reagent was transferred to the respective plates preloaded with standards and unknown ovalbumin. After the addition of 2N Folin, the OD was read at 0^{th} min only. The readings with fresh reagent were taken for stability comparison while reagent kept at RT was considered as negative control.

Validation of stored reagent with biofluids: Twenty-five serum samples were diluted to 1 mg/ml based on theoretical calculation. Five microlitres of diluted samples were estimated by different methods: Fresh Lowry reagent- 1N Folin (at 20 min), 2N Folin (at 0^{th} min) and stored Lowry reagent (at 0 °C for 2-, 4-, 15- and 30- days) followed by 2N Folin at 0^{th} min. The OD was recorded at 620nm after respective incubation time. The protein concentration in neat serum was back calculated and expressed in mg/ml.

CSF samples: The protein in 20 µl of CSF samples was quantified by improved protocol (2N Folin at 0^{th} min) with fresh and stored (for 30 days) Lowry reagent. The estimated protein contents were expressed in mg/ml.

Standard curve and extrapolation: The optical densities were automatically plotted against protein content and then a best fit trendline (with R^2 value) was constructed for each method by automated interfaced SkanIt™ software (Thermo scientific™). For the improved method, a linear curve was drawn separately for each time points with their respective BSA standards. The conventional (1N Folin), improved protocol (2N Folin) at different time points were compared based on regression co-efficient (R^2) of standard curve. For reagent stability and validation assay, the elements in linear equation, %CV of OD and the estimated protein content (mg/ml) were tabulated and compared.

Statistical analysis: Initially, the intra-assay variation in optical density (OD) was calculated by percentage co-variance (%CV) and the linearity was compared by regression co-efficient (R^2). Bland Altman plot was created to display the mean difference of the known ovalbumin estimated by the conventional method (2N-20 min) and the modified 0^{th} min readings (Y-axis) to the average of ovalbumin (X-axis), corresponding to the level of agreement between the two tests. The proposed rapid method at different experimental setup was validated by comparing RSE, slope value and the mean protein content. Statistical significance at $P<0.05$ was analyzed using data analysis tool kit and real statistics pack in MS office excel v2010.

Results

Comparison of absorbance and estimated protein: The OD taken after 20 min of 1N Folin exhibited a polynomial linear line whereas the 0^{th} min readings of 2N Folin fitted in a linear regression model (Figure 1). On comparison at 20 min, the conventional 1N Folin (4.04 %CV) was similar to that of undiluted 2N (4.72 %CV) method. The cumulative intra-assay and inter-assay %CV of 2N Folin 0^{th} min was 4.39% which is well within the acceptance range of <10%.

The estimated protein level in different dilutions of ovalbumin by 1N Folin at 20^{th} min showed concurrence with improvised method at all-time points.

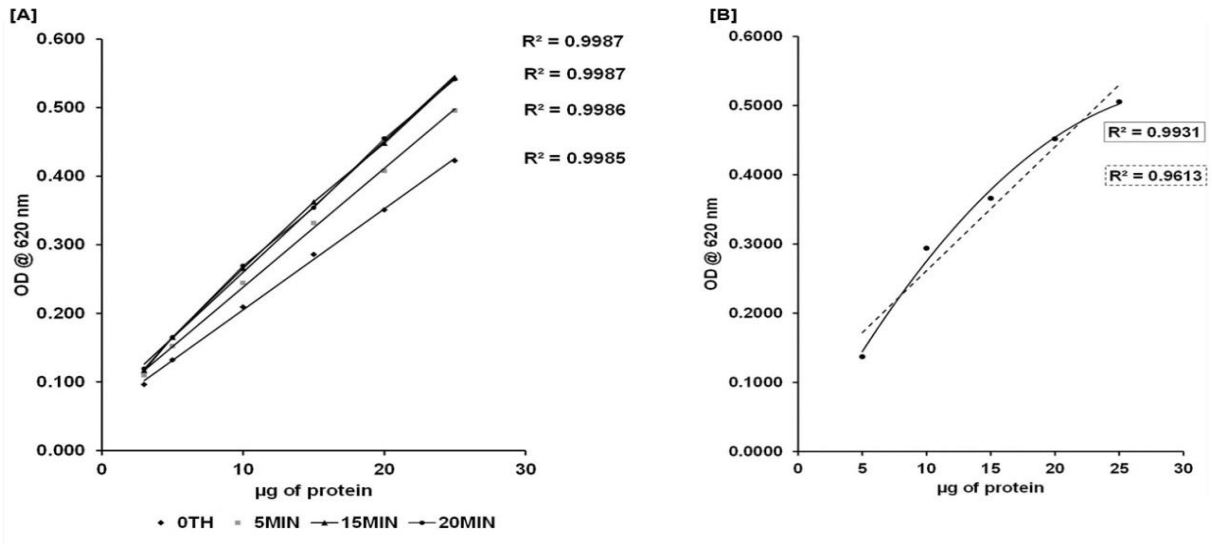

Figure 1: Comparison of standard curve between 2N and 1N Folin

Note: Trendline of [A] improved (2N) and [B] conventional (1N-20 min) Lowry protocols. The dashed line (right) represents the linear fit while polynomial curve was depicted in solid

Once incubation time was extended from 0 to 20 min, an increase in the OD values was observed in 2N Folin. However, the Bland-Altman plot for unknown ovalbumin (n=50) estimated in each instance does not significantly differ between 20th and 0th min (Figure 2). This was evidenced by nearly 95% of values which are closer to the mean bias value. The correlation matrix suggests that there is a strong correlation (r=0.973) between estimated values of serum protein at 0 and 20 min. The best fit for regression line was compared by R^2 and slope value while the predictions given by individual regression model were validated by residual standard deviation (RSE) (Table 1).

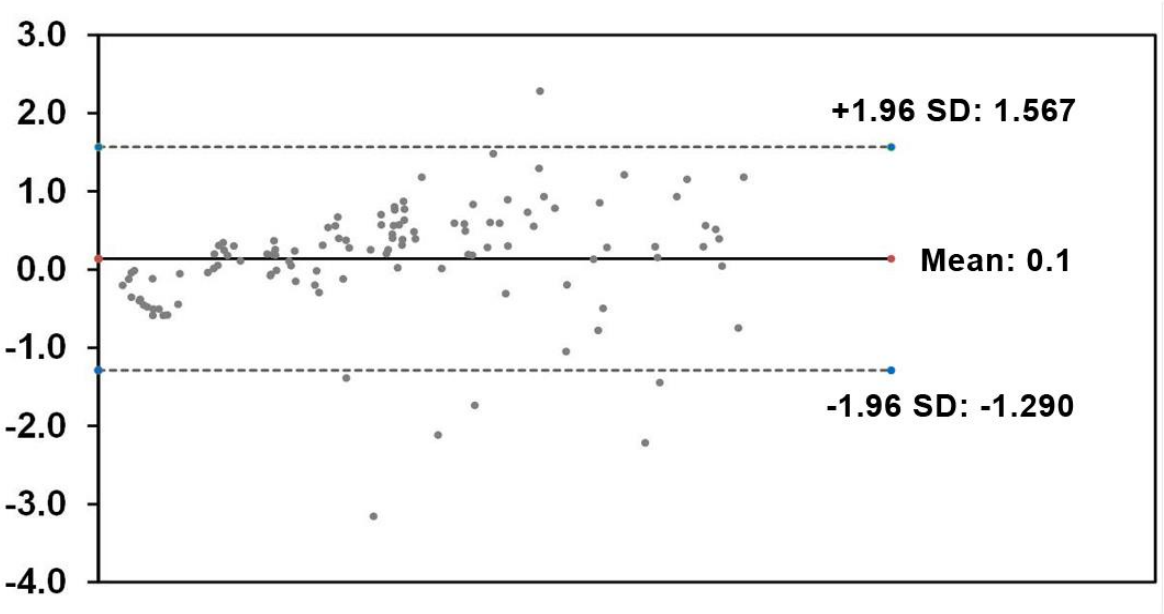

Figure 2: Bland Altman plot for comparison of estimated proteins

Note: In Bland Altman plot, each dot represents individual unknown ovalbumin protein estimated by two methods; 2N-20 min and 2N-0 min. The solid horizontal line denotes the mean bias between the two methods. The dashed horizontal lines indicate the standard deviation from the mean (solid line) with 95% limits of agreement

Table 1
Comparison of undiluted 2N Folin at different time points

Assay plate	Date	RSE	R2	M	RSE (mean±SD)	R2 (mean±SD)	M (mean±SD)
Plate 1	Direct 20 min	0.006	0.999	0.02035	0.009±0.003	0.996±0.002	0.0191±0.004
	Direct 20 min	0.010	0.995	0.01462			
	Direct 20 min	0.012	0.995	0.02234			
Plate 2	0MIN	0.003	0.999	0.01565	0.005±0.001	0.997±0.001	0.01456±0.003
	0MIN	0.005	0.999	0.01405			
	0MIN	0.007	0.995	0.01055			
	0MIN	0.006	0.998	0.01801			
	7MIN	0.004	0.999	0.01946	0.007±0.001	0.997±0.001	0.0177±0.004
	7MIN	0.008	0.999	0.01876			
	7MIN	0.008	0.995	0.01138			
	7MIN	0.007	0.998	0.02117			
	14MIN	0.004	0.999	0.02136	0.008±0.002	0.997±0.002	0.0195±0.004
	14MIN	0.010	0.999	0.02030			
	14MIN	0.008	0.995	0.01235			
	14MIN	0.010	0.997	0.02384			
	20MIN	0.005	0.999	0.02211	0.007±0.003	0.997±0.002	0.0202±0.005
	20MIN	0.004	0.999	0.02111			
	20MIN	0.009	0.995	0.01271			
	20MIN	0.011	0.997	0.02488			
Cumulative (mean±SD)					0.007±0.002	0.997±0.002	0.01798±0.006

Note: RSE-residual standard error; R2-correlation co-efficient; M-slope

Table 2
Comparison of fresh and stored Lowry reagent

Days of storage	Conditions	RSE	R2	M
DAY 2	SR-2D-Z	0.0041	0.999	0.015172
	SR-2D-T	0.0752	0.850	0.019643
	SR-2D-E	0.0269	0.977	0.019141
DAY 4	SR-4D-Z	0.0085	0.990	0.021552
	SR-4D-T	0.0200	0.985	0.017307
	SR-4D-E	0.0260	0.984	0.021625
DAY 15	SR-15D-Z	0.0075	0.982	0.017949
DAY 30	SR-30D-Z	0.0150	0.989	0.016386
Fresh reagent (2N)		0.0064	0.998	0.016058

Note: SR-stored reagent; FR- Fresh reagent; D-days; Z-0 ºC; T-20 ºC; E-80 ºC; N-normality.

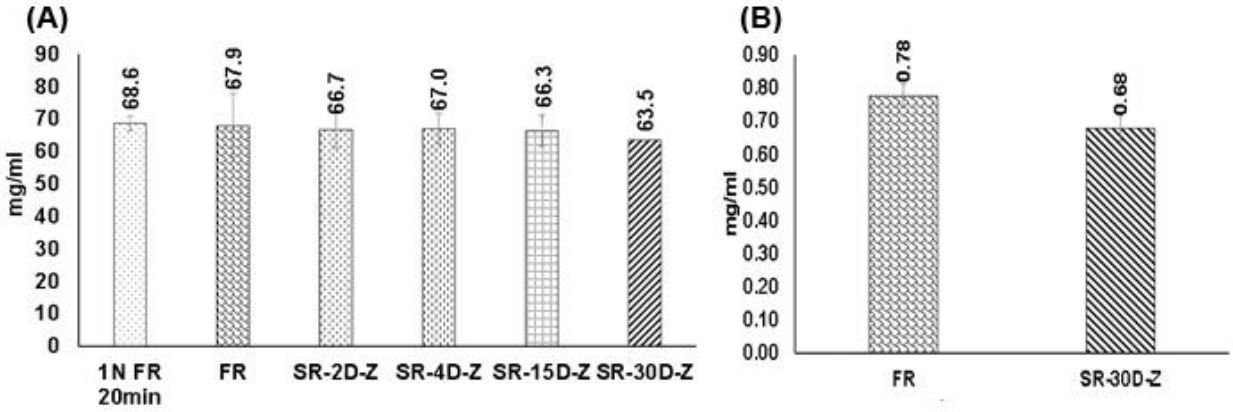

Figure 3: Comparison of total protein between fresh and stored Lowry reagent
Note: (A) serum samples (B) CSF samples. Serum total protein was back calculated from estimated value and both were expressed in mg/ml (mean±SD). The stored 5x Lowry reagent (SR) at 0 ºC (Z) for up to 30 days (D) was compared with fresh reagent (FR).

Comparison of fresh versus stored Lowry reagent: Initially, RSE and slope were analyzed across the 5X Lowry reagent kept at 0 ºC, -20 ºC and -80 ºC for 30 days. Kendall's tau correlations of stored reagent showed strong agreement (r>0.95) with fresh reagent, except reagent stored at -80 ºC. Hence for validation assay, aliquots of 5X reagent at 0 ºC were used to estimate protein content in biofluids. The difference in total protein concentration in human serum was depicted in figure 3. The protein in serum samples estimated by conventional method ranged from 68.6±2.2 mg/ml. The proposed rapid 10 min protocol was akin to 20 min estimation with a concentration of 67.9±9.9 mg/ml. ANOVA with post-adhoc test also indicated that there was no statistical difference between mean protein level between fresh and Lowry reagent kept at 0 ºC for up to 30 days.

Discussion

At first, the OD and estimated proteins by diluted Folin method were compared with different time points of 2N Folin method. Multiple statistical tools such as R^2, RSE, slope, ANOVA were employed to conclude that there was no significant variation in the results of former and latter protocols. Hence, all assays on stored Lowry 5X reagent were read on 0^{th} min thereafter and compared with the 0^{th} min of freshly prepared reagent. Earlier the standard curve was meant to be non-linear with the BSA range of 3-300 ug/ml[2]. This method yielded a linear trendline with BSA (30 to 250 ug/ml) with mean R^2 of 0.998. This microtitre assay offers serial dilutions of unknown samples much easier to avoid the trial protein range estimations.

The undiluted 2N Folin was attempted before quantifying Leishmania proteins in a total crude extract or in a soluble fraction[1]. However, the readings were taken after 30 min incubation whereas the current protocol was optimized for immediate (0^{th} min) reading. Modified method by replacing copper nitrate instead of copper sulphate also improves lower limit of detection and reduces time from 40 to 33 min[9]. Besides, a research group tried the addition of sodium dodecyl suphate or solvents for destroying bubbles or turbidity produced in the final reaction mixture.

The findings of this study recommend the use of stored alkaline-copper-tartrate reagent preferably at 0 °C. Hence, there is no need of chemical preparation every time and this will reduce reagent induced variations. An attempt was made by replacing carbonate buffer (pH 11.4) with phosphate buffer (100 mM) to achieve 25% increase in sensitivity, that

too with reagent stability up to 2 weeks[8]. In this study, instead of changing the reagent recipe, the premixed solution was frozen for up to one month. The scattered plot showed strong correlation with the OD values of fresh reagent and the overall protein content in serum and CSF samples.

Conclusion

The variations observed in the absorbance between 1N and 2N Folin do not reflect in estimated value and total proteins concentration in serum samples. This improvised that rapid Lowry method (10 min) of protein estimation is beneficial to resource limiting clinical and research laboratories. The utility of statistical tools such as residual standard error for validating precision of predictors and Bland Altman plot for comparing measures between two methods was recommended. It was evidenced that the 5X reagent can be stored at 0 °C for 30 days without any significant changes in protein estimation.

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