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Significance of method validation/verification: estimation of total proteins in cerebro spinal fluid by 3%trichloro acetic acid method in limited resources areas

[1]Dr. Sasikala .T, [2]Dr. N. Lakshmanna, [3]Dr. D. Christy Pradeep Raj, [4]Dr. N. Viswabharathi

Abstract: ***Background*:** The validation of analytical methods and the calibration of equipment are of the essencial aspects of quality assurance programs in the laboratory. All the methods used in an analytical chemistry laboratory must be evaluated and tested to ensure that they produce valid results suitable for their intended purpose i.e. they must be validated. The commonly used 3% sulphosalicylic acid method for the determination of total proteins in cerebro spinal fluid does not give satisfactory results. Hence the present study aims to study and evaluate the method for the estimation of proteins in CSF by 3% TCA. ***Methodology:*** 3%Trichloroaceticacid (TCA) method was used for estimation of total proteins in cerebro spinal fluid by colorimeter and spectrophotometer. ***Results:*** Within run precision check revealed that the coefficient of variation was 1.57%. In the present study 4.76mg of protein was added and observed the recovery of the analyte. Recovery percentage was calculated by formula i.e concentration of recovered protein divided by concentration of protein was added multiplied with hundred. We found percentage of recovery was 147%. ***Conclusion:*** We found that trichloroacetic acid method was linear up to 150mg/dL. Hence the observations of the present study concluded that 3% TCA method was simple precised and useful method to estimate total proteins in cerebrospinal fluid. Consideration of the financial constraints, this method can be very useful in resource limited centres.

Keywords: Trichloro acetic acid, Coefficient variation, Precision, Recovery.

AFFILIATIONS
[1]*Assistant Professor, Department of Biochemistry, Government Medical College, Eluru.*
[2]*Assistant Professor, Department of Biochemistry, Government Medical College, Kurnool.*
[3]*Assistant Professor, Department of Biochemistry, Government Medical College, Eluru.*
[4]*Associate Professor, Department of Biochemistry, Government Medical College, Eluru.*

CORRESPONDING AUTHOR
Dr.N. Viswabharathi,
viswabharathi.nalla@gmail.com

INTRODUCTION

The validation of analytical methods and the calibration of equipment are of mandatory aspects of quality assurance in the laboratory. All analytical methods used in laboratory requisite tobe evaluated and tested to ensure that they produce valid results suitable for their intended purpose. All the methods must be validated (1). New methods are routinely introduce to produce quality reports. Whatever novel methodologies introduced into a laboratory should also be documented and all experts who will exercise it must acquire adequate training and demonstrate their competence in the method before commencing actual casework. Commercial methods also need revalidation, or at least verification. Method evaluation and recurrent recovery experiments will be helpful to standardise a method where reference method is unavailable and the resource limited centers were there in rural areas especially developing countries like India. 3% Tri chloroacetic acid method was simple one of the methods for quantify proteins in cerebro spinal fluid. Amongst the methods for the determination of total protein in cerebro spinal fluid, that involving a 3% sulphosalicylic acid solution and 3% Tri chloroacetic acid (TCA) were commonly used owing to its simplicity and rapidity. There exists, however, a great discrepancy between the total protein found in an artificial albumin and globulin mixture when the TCA and the sulphosalicylic acid methods are compared (1, 2). The commonly used 3% sulphosalicylic acid method for the determination of total proteins in cerebro spinal fluid (CSF) does not give satisfactory results. Hence the present study aims to study and evaluate the method for the estimation of total proteins in CSF by 3% TCA method.

MATERIALS AND METHODS

The study was conducted to evaluate the quality of the method in the department of Biochemistry, NRIIMS college, visakhapatnam during 2022 after obtaining IEC approval. CSF samples were collected from outpatient departments of general medicine. Total proteins was estimated in cerebro spinal fluid by 3%Trichloro acetic acid (TCA)method. Stadardisation and presission and recovery experiments will be performed to check the linearity, Limit of detection (LOD), sensitivity and specificity.

Statistical analysis: Data was entered in the excel spread sheets. Standard curve graphs was performed. Data of precision and recovery experiments entered and standard deviation and coefficient of variation was performed.

METHODOLOGY

3%Trichloro acetic acid (TCA) method for estimation of total proteins in cerebro spinal fluid

Principle: TCA removes the shell of hydration from proteins in CSF and gets precipitated. Scattering is measured in colorimeter which is directly proportional to concentration of protein levels in the CSF

Reagents : 3% TCA: Dissolve 3.0g TCA in 100 mL of distilled water

Standard Concentration: 50mg/dL (weigh 50mg of protein dissolve in 100mL of distilled water)

Procedure:

Take 3 clean and dry test tubes and labelled them as blank standard and test.

Add 1mL of distilled water, Standard solution, CSF sample to Blank, Standard and Test test tubes respectively.

Add 4mL of 3% TCA solution to all the above tubes.

Mix well and incubate for 10mintutes. Measure the scattering absorbance at 403nm. The absorbance is directly proportional to concentration of Protein in the CSF sample.

RESULTS

Table 1: Procedure of estimation of total proteins in CSF

Pipette into tubes	Blank	Standard	Test
Distilled water	1mL	-	-
Standard	-	1mL	-
CSF sample	-	-	1mL
TCA reagent	4 mL	4 mL	4 mL

Incubate for 10minutes and measure the absorbance at 403nm.

Calculations :

Concentration of protein in CSF is (mg/dL): Abs of Test/Abs of Standard *50

Standardisation: working standards were prepared from stock standard solution showed in table 2.

Table 2: working standards preparation of total proteins in CSF

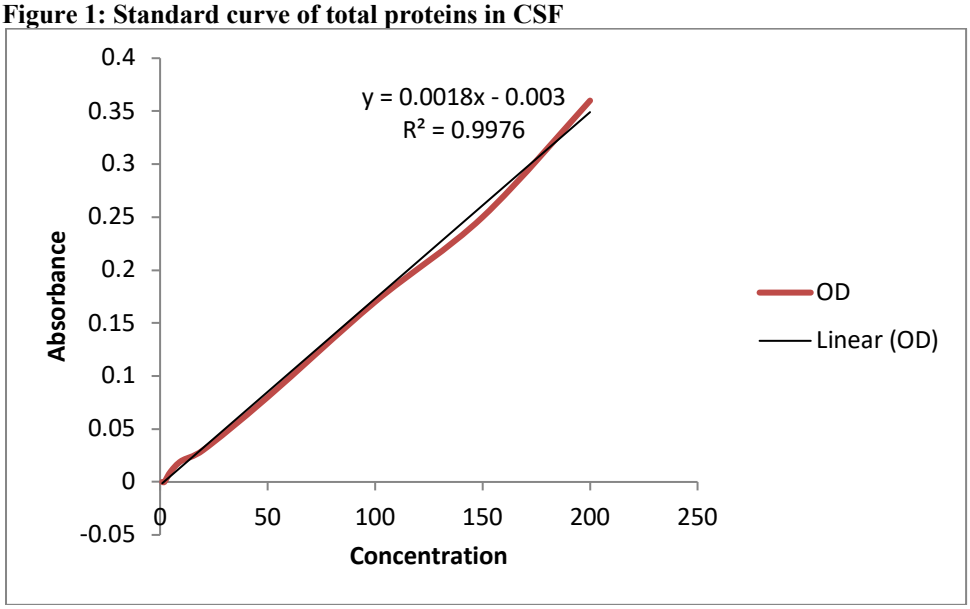
Required Concentration	Stock (200mg/dL)	Distilled water (mL)	Final Volume (mL)	Absorbance
1.0 (mg/dL)	10 µL	1.99	2.0	0.0
2.0 (mg/dL)	20 µL	1.98	2.0	0.0
5 (mg/dL)	50 µL	1.95	2.0	0.01
10 (mg/dL)	100 µL	1.9	2.0	0.02
20 (mg/dL)	200 µL	1.8	2.0	0.03
50 (mg/dL)	0.5mL	1.5	2.0	0.08
100 (mg/dL)	1.0mL	1.0	2.0	0.17
150 (mg/dL)	1.5mL	0.5	2.0	0.25
200 (mg/dL)	2.0	-	2.0	0.36

Table 3: standard concentrations and obtained values

Standard Concentration	Absorbance	Value
1.0 (mg/dL)	**0.0**	**0.0**
2.0 (mg/dL)	**0.0**	**0.0**
5 (mg/dL)	**0.01**	**6.25**
10 (mg/dL)	**0.02**	**12.5**

20 (mg/dL)	**0.03**	**19**
50 (mg/dL)	**0.08**	**50**
100 (mg/dL)	**0.17**	**106**
150 (mg/dL)	**0.25**	**156**
200 (mg/dL)	**0.36**	**225**

Figure 1: Standard curve of total proteins in CSF



Linearity: 5-100mg/dL
Limit of detection (LOD): 5mg/dL

Table 4: Within run precision-CSF total proteins:

Value (x)	Deviation from mean (X-X= x) —	Square of deviation (x^2)
19.5	0.2	0.04
19	0.7	0.49
19.5	0.2	0.04
20	-0.2	0.04
19.3	0.4	0.16
19.8	0.07	0.0
20.1	-0.3	0.06
20	-0.2	0.04
20	-0.2	0.04
20.1	-0.3	0.06
197.3/10=19.73		0.97

$$s^2 = \hat{\sigma}^2 = \frac{\sum (X_i - \overline{X})^2}{(n-1)}$$

Variance (s^2) =

S^2 =0.97/10-1= 0.10

Standard Deviation (SD) = $\sqrt{S^2}$ =$\sqrt{0.1}$= 0.31

Coefficient of variation (CV) = SD/Mean × 100

= 0.31/19.73 X 100

1.57%

Recovery Experiment:

The proportional error and its magnitude is estimated by recovery experiment. We have taken clean dry test tubes and labeled them as base line (B) and spike (S). For both tubes 2mL of sample was added and in "B" 100µL of distilled water added and 100µL of stock standard to "S" tube. To another two tubes, labeled as Blank and Test and performed the estimation by using the reagents as normal procedure and base line and spike were taken and calculated.

Table 5: Sample preparation for recovery experiment

Pipette into tubes	Baseline	Spike
CSF	2mL	2mL
Distilled water	100µL	-
Standard Concentration	-	100 µL

Table 6: Procedure of recovery experiment by TCA method

Pipette into tubes	Baseline	Spike
TCA reagent	4mL	4mL
Sample	1 mL	1 mL
OD	0.17	0.18

Table 7: recovery of the experiment

parameter	Protein added	Protein measured	Recovery
Baseline		106	-
Spike	4.76	113	07

$$\textbf{\% Recovery} = \frac{\text{Conc. of recovered}}{\text{Conc. of added}} \times 100$$

% Recovery = 07/4.76*100

147%

DISCUSSION

To evaluate a method require the following set of validation parameters to be determined such as specificity/selectivity, limit of detection (LOD), precision and accuracy (bias), linearity and working range, recovery, uncertainty of measurement, stability.

*Specificity :*It is a measure of the ability of the method to identify/quantify the analytes in the presence of other substances, either endogenous or exogenous, in a sample matrix under the stated conditions of the method (2, 3).

*Limit of detection (LOD):*This is the lowest analyte concentration that can be detected and identified with a given degree of certainty. The LOD is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence. The LOD is not a robust factor and can be affected by minor changes in the analytical system such as temperature, purity of reagents, matrix effects, and instrumental conditions. It is therefore important that this parameter is always verified by laboratories adopting previously validated methods (1-4). In this study we observed that 3% TCA method linear up lo 150mg/dL in CSF after that the method showed significantly increased values compared with true value. In this study we observed that Colorimetric reading was not observed below 5mg/dL concentrations such as 1 mg/dL and 2 mg/dL. The present study was derive LOD of 3%TCA method for CSF protein was 5mg/dL (table 3 and Fig 5).

Precision: Two commonly accepted sets of conditions under which precision is measured are repeatable and reproducible conditions. Repeatability conditions occur when the same analyst analyses samples on the same day with the same instrument in the same laboratory. Any variation from these conditions (e.g. different analysts, different days, different instruments, different laboratories) represent reproducibility conditions. Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results obtained from independently prepared quality control standards (5). Acceptable precision at the lower concentrations is 20%. The present study was done precision experiments with ten samples. We observed that within run precision check of 3% TCA method showed that standard deviation (0.3) and better percentage of coefficient of variation (1.57%)

Recovery: The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the matrix, compared to the detector response for the true concentration of the pure authentic standard or seized materials. It may also be understood as the percentage of the drug, metabolite, or internal standard originally in the specimen that reaches the end of the procedure. In the case of biological specimens, blanks of the biological matrix once the final extracts have been obtained may be spiked with the true concentration of the pure authentic standard and then analysed. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (typically those corresponding to control samples used to evaluate a method's precision and accuracy). Recovery of the analyte not necessary to be 100%, but the magnitude of recovery should be consistent, precise and reproducible (1-5).

TCA (3%) were described in the present study. This method consists of the use of a 3% trichloroacetic acid solution to estimated CSF proteins. In the present study 4.76mg of protein was added and observed the recovery of the analyte. Recovery percentage was calculated by formula i.e concentration of recovered protein divided by concentration of protein was added multiplied with hundred. We found that the coefficient of variation was 1.57% and percentage of recovery was 147%. We observed that this method showed better coefficient of variation an as well as recovery also.We found that this method was linear up to 150mg/dL.

CONCLUSION

The present study concluded that 3% TCA method was simple, precised and useful method to estimate total proteins in cerebrospinal fluid. Consideration of the financial constraints, this method can be very useful in resource limited centres.

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