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EVALUATION OF TURBIDIMETRIC BENZALKONIUM CHLORIDE METHOD FOR ESTIMATION OF CSF PROTEIN.

Pathology

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ABSTRACT

Background: Cerebrospinal fluid (CSF) protein estimation is an important routine diagnostic tool for many diseases. Various methods have been used to measure protein in CSF. The most common methods used for the measurement of total protein concentration in CSF are turbidimetry of precipitated proteins or spectrophotometry of protein-bound dyes.
Material and Methods: Hundred non-blood stained CSF samples were analysed by both 3% TCA and BKC methods.
Results: Coefficient of variation (CV) for TCA method was 3.38% and for BKC was 3.66%. Repeatability coefficient for BKC was 0.013 and for TCA was 0.030. For reliability, Intraclass correlation coefficient for BKC was 1.00 [95% CI 1.000 – 1.000] and for TCA was 0.999 [95% CI 0.998 – 1.000]. The within-subjects CV for BKC was 1.68% and for TCA was 6.77%. The Pearson correlation coefficient (r) was 0.99.
Conclusion: Protein values of BKC and TCA method correlate well. In imprecision studies, the repeatability, reproducibility and reliability of BKC method was better than that of TCA. BKC method is quick, simple, cost effective and precise, with advantage of being automated.

KEYWORDS

CSF protein estimation, 3%Trichloroacetic acid, Benzalkonium chloride, Imprecision studies

INTRODUCTION

Cerebrospinal fluid (CSF) is a clear, colourless fluid that surrounds brain and spinal cord. It provides a basic mechanical and immunological protection to the brain inside the skull by acting as a cushion or buffer for the cortex.[1] Measurements to determine alterations in the composition of CSF are central in the differential diagnosis of specific diseases of the central nervous system (CNS). In particular, the analysis of the CSF protein composition provides crucial information in the diagnosis of CNS diseases. The protein value of CSF equals 0.3% plasma proteins or approximately 15 to 40mg/dl, depending on sampling site.[2]

CSF protein can be determined by turbidimetric assays using trichloroacetic acid (TCA)[3], sulfosalicylic acid (SSA)[4], benzethonium chloride (BTC)[5,6] or Benzalkonium chloride (BKC)[7,8]; or protein dye binding assays using Coomassie Brilliant Blue (CBB)[9], Pyrogallol Red-molybdate (PRM)[10,11] or Ponceau-S[12]. Many different methods for measuring total protein in CSF have been described indicating that no single method overcomes all the associated problems. The disadvantage of dye binding methods is that different proteins yield coloured products with different molar absorbencies; and some of these methods also involve tedious precipitation and redissolving which is inconvenient if many samples are to be processed. In turbidimetric methods, precipitating agents which are being used do not always precipitate different proteins to the same extent. A quick, simple, sensitive and inexpensive method is required which also gives similar response to each of the various proteins in CSF. A wide range of investigations are performed on CSF samples usually received in small quantities in the laboratory, hence, a method requiring less sample volume is preferred.

Shephard and Whiting were among the first ones to use BKC as a precipitating agent for the determination of protein in CSF by automated nephelometry method. BKC showed good precision and closest agreement with the TCA-Ponceau S dye binding method.[7] However, many clinical laboratories lack access to automation due to cost implications.

The comparison of two different analytical methods that measure the same analyte can be assessed by method comparison studies for agreement. TCA is being used since early 19th century as conventional method. To the best of our knowledge there was no study comparing BKC manual method with the more commonly used 3%TCA manual method. We hypothesize that both the methods are agreeable and hence, manual turbidimetric method using BKC was compared with 3% TCA conventional manual method.

MATERIALS & METHODS

Institutional ethical committee approval was obtained before commencing the study. This study was carried out in the Department of Pathology of a tertiary care teaching hospital from January 2019 to October 2019. The CSF samples received in department for routine testing were included in the study. All grossly haemorrhagic samples were excluded from the study.

Methodology

Total 100 samples were analysed by both BKC and TCA manual methods. Reagents used for both methods were locally purchased from Loba Chemie Pvt. Ltd. and protein standards were purchased from Transasia Bio-Medicals Ltd. Instruments used were Kanad Vidyut colorimeter and pipettes for measuring the stated volumes. Pipettes were calibrated by the gravimetric and volumetric method, so as to avoid pipette calibration error. Pipette tips of appropriate size and cuvettes were used. A standard protein solution (6gm/dl i.e. 6000mg/dl) was used as a stock standard. Working protein standard was made by 1/10th (600mg/dl) and 1/100th (60 mg/dl) dilution of stock protein standard.

Protein Estimation by Benzalkonium Chloride (BKC)[13]: BKC is a detergent and causes protein denaturation in an alkaline medium. Reagents used were NaOH/EDTA reagent (reagent 1) containing 33mmol/L EDTA and 0.5mol/L NaOH; and the BKC solution (5g/L; reagent 2) which was prepared from a 50g/L aqueous stock solution of BKC. In manual procedure, 1.0mL of NaOH/EDTA was mixed with 100µl of sample and blank reading was taken (A1), then, immediately 250µl of Benzalkonium Chloride reagent was added. After 10min incubation, the absorbance (A2) at 630nm in a colorimeter was measured. After subtracting A1 from A2, these reading were used for calculations.

Protein Estimation By 3% Trichloroacetic acid (TCA)[14]: Reagents used were 3% TCA (3percent w/v): 30g of crystalline Trichloroacetic acid per litre in water was taken to make 3% TCA. For test sample 200µl of CSF was mixed with 500µl of 3% TCA. After 10min, reading of the optical density of the solution at 630nm was taken.

For comparison of both methods, Standard Deviation (SD) and Coefficient of Variation (CV) were calculated. A known standard protein concentration of 60mg/dl was taken in 20 different test tubes and its absorbance was measured in digital colorimeter by both BKC and TCA methods under similar conditions. To see for the linearity of BKC and TCA, standard protein concentrate was serially diluted to get protein values ranging from 6mg/dl to 600mg/dl and graphs were plotted with the absorbance values of each protein concentration by both the methods under similar conditions. In imprecision studies, repeatability, reliability and reproducibility were calculated. For

repeatability known concentration standard proteins were taken and their absorbance was calculated at the end of 0hr, 1hr, 2hr and 3hrs. From these values repeatability coefficient was calculated.

Determination of factor: In order to estimate protein values by two different methods, the equation is as follows, CSF Protein = Absorbance × Factor. CSF protein value is the value assigned to the standard protein concentration and absorbance is the direct value which can be read from colorimeter. Hence, factor is "known or standard protein value divided by the absorbance value". Thus, factor value was derived for each of the two methods.

STATISTICALANALYSIS

Data was entered into MS Excel spreadsheets and analyzed using SPSS Statistics version 21. Quantitative data such as age, absorbance and CSF protein was summarized using mean ± SD where data was normally distributed or median (Inter-quartile range) where data was skewed. To test the difference between proportions Chi square test (χ^2) was used. Pearson correlation (r) coefficient was used to correlate between CSF proteins as estimated by TCA and BKC. Repeatability coefficient was calculated for absorbance by both methods. Reliability was measured using Intraclass correlation coefficient. Linearity for both methods was plotted using scatter plot. Variability in the measurements was measured using within-subject coefficient of variation. For method comparison, Imprecision was reported in terms of CV and agreement was assessed using Bland and Altman plots. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Protein estimation of total 100 CSF samples was done using both the methods. The characteristics of the CSF samples used in our study are given in Table 1. The median of CSF protein calculated by 3% TCA was 60.75mg/dl (IQR 37.70 – 165.50) [Range 9.9mg/dl - 499.2mg/dl] and that measured by BKC was 59.05mg/dl (IQR 29.60 – 155.4) [Range 8.4mg/dl - 481.6mg/dl]. The difference between them was statistically significant (Z=6.59, p<0.001). The distribution of CSF protein values according to age is shown in Table 2.

Table 1: Characteristics of the CSF samples (n = 100).

Variable	Frequency (%)
Age	
< 1 month	15 (15%)
1 month – 3 months	9 (9%)
3 months – 6 years	21 (21%)
6 years – 12 years	13 (13%)
12 years – 50 years	26 (26%)
> 50 years	16 (16%)
Gender	
Male	57 (57%)
Female	43 (43%)
Color	
Colorless	81 (81%)
Whitish	10 (10%)
Yellowish	7 (7%)
Pale yellow	2 (2%)
Appearance	
Clear	75 (75%)
Slightly hazy	11 (11%)
Hazy	8 (8%)
Turbid	6 (6%)

Table 2: Age-wise distribution of CSF protein values obtained by both TCA & BKC methods (n=100).

Protein Values / Age group	< 15mg/dl		15-40mg/dl		>40mg/dl	
	TCA	**BKC**	**TCA**	**BKC**	**TCA**	**BKC**
< 1 month	1(1%)	1(1%)	1(1%)	1(1%)	13(13%)	13(13%)
1 month – 3 months	0(0%)	0(0%)	3(3%)	3(3%)	6(6%)	6(6%)
3 months – 6 years	0(0%)	1(1%)	6(6%)	6(6%)	15(15%)	14(14%)
6 years – 12 years	1(1%)	2(2%)	4(4%)	3(3%)	8(8%)	8(8%)
12 years – 50 years	0(0%)	3(3%)	8(8%)	7(7%)	18(18%)	16(16%)
> 50 years	0(0%)	0(0%)	3(3%)	3(3%)	13(13%)	13(13%)
Total	**2 (2%)**	**7 (7%)**	**25 (25%)**	**23 (23%)**	**73 (73%)**	**70 (70%)**
	χ^2 = 2.250, P = 0.522		χ^2 = 0.126, P = 0.999		χ^2 = 0.089, P = 0.999	

Linearity and CV: Absorbance of known protein concentrates ranging from 6mg/dl to 600mg/dl were plotted on a graph and used for linearity [Figure 1 and Figure 2]. TCA method showed a good linearity from 6mg/dl to 600mg/dl, however, for BKC a good linearity was observed till protein concentration of 420mg/dl. For protein values more than 420mg/dl, volume of the sample was halved and separate factor was calculated from which protein value of test sample was calculated. For TCA, the mean absorbance value was 0.110 ± 0.0037 [95% CI 0.102 – 0.116]. CV for TCA method was 3.38%. For BKC, the mean absorbance value was 0.259 ± 0.0095 [95% CI 0.241 – 0.277]. CV for BKC method was 3.66%.

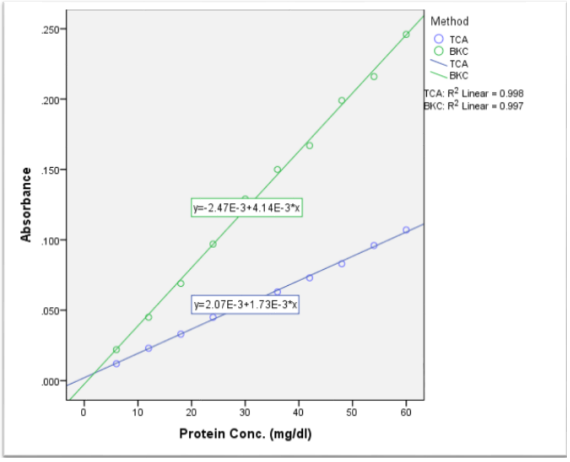


Figure 1: Comparison of absorbance and protein concentrations of BKC and TCA from 6mg/dl to 60mg/dl.

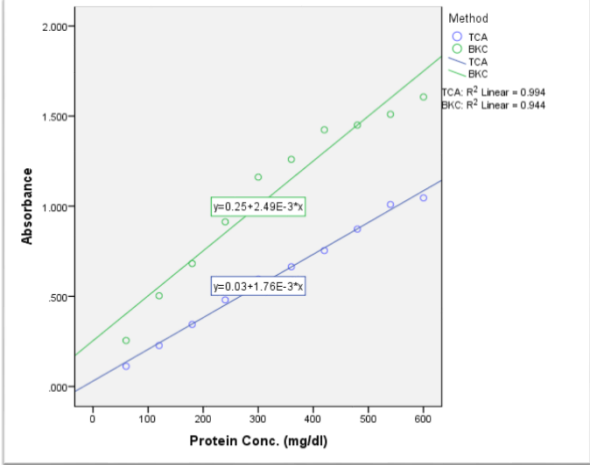


Figure 2: Comparison of absorbance and protein concentrations of BKC and TCA from 60mg/dl to 600mg/dl.

Imprecision Studies:

Repeatability coefficient for BKC was 0.013 and for TCA was 0.030. For reliability, Intraclass correlation coefficient for BKC was 1.00 [95% CI 1.000 – 1.000] and for TCA was 0.999 [95% CI 0.998 – 1.000]. The within-subjects CV for BKC was 1.68% and for TCA was 6.77%.The reproducibility coefficient was measured from protein values of 100 CSF samples and found out to be 11.79 and the reproducibility limit was 32.67.

The agreement between two methods was depicted using Bland and Altman plots [Figure 3] with mean differences plotted on y-axis and means on x-axis. The methods show greater variability as the protein concentration increases. For bias, the mean difference between protein measured by TCA and BKC was 8.370 [95% CI 5.06 – 11.68]. TCA tends to give higher protein concentration and the difference was statistically significant (t = 5.016, P<0.001).

The Pearson correlation coefficient (r) between two sets of data was 0.99 with a 95% CI of 0.986 – 0.993. The correlation was found to be statistically significant (P<0.001) [Figure 4].

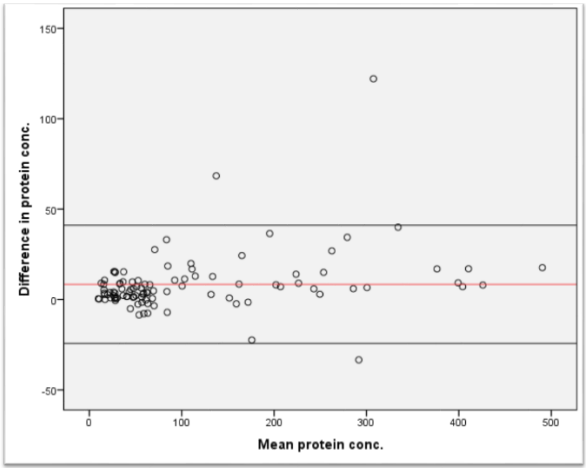


Figure 3: Bland-Altman plot for agreement between BKC and TCA (n=100).

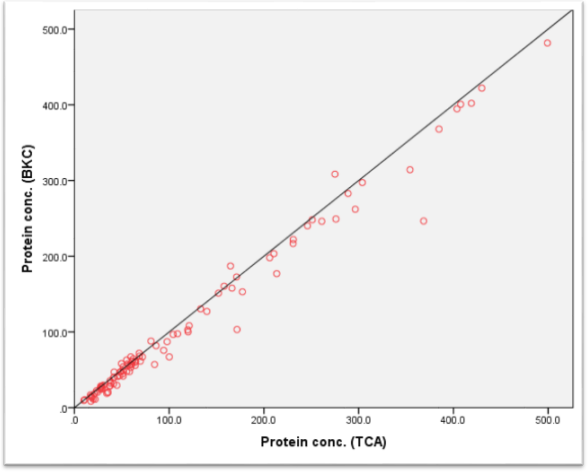


Figure 4: Correlation between BKC and TCA for CSF protein (n=100).

Out of all 100 CSF samples, 21 samples showed >10RBCs/hpf on microscopic examination, which when measured by TCA showed higher protein values than BKC. On gross examination, 2 samples were yellowish and 19 samples were colourless. The mean protein value measured by TCA was 122.76mg/dl [Range 67.2mg/dl - 258.6mg/dl] and that of BKC was 76.83mg/dl [Range 25.7mg/dl - 203.4mg/dl]. The mean difference between protein values by both methods was 45.93mg/dl, which was statistically significant. [t = 9.157, P<0.001].

DISCUSSION

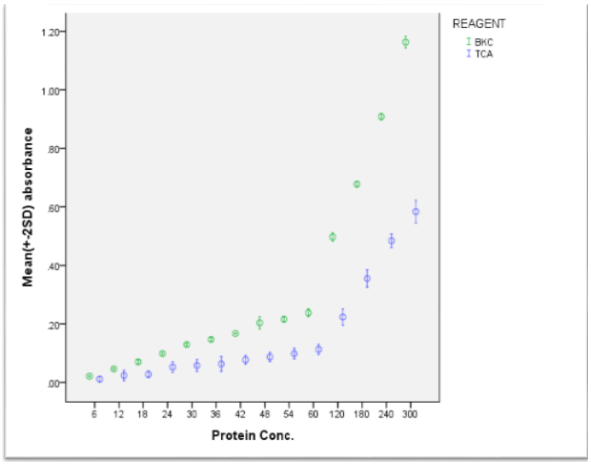
The present study showed that protein values of BKC and TCA method correlate well. In imprecision studies, the repeatability, reliability, and reproducibility of BKC method was better than that of TCA. CSF samples with presence of RBCs (hemoglobin) lead to overestimation of protein concentration when assayed by TCA but using BKC, measurement of protein concentration was minimally disturbed. Though TCA manual method had good linearity and correlation with BKC manual method for protein estimation, it showed poor reproducibility, repeatability and reliability. Moreover, the sample volume required by BKC was half to that of TCA.

Shephard and Whiting used four different protein precipitants trichloroacetic acid (TCA), sulphosalicylic acid (SSA), benzethonium chloride (BTC) and benzalkonium chloride (BKC) to estimate the total protein concentration in CSF by automated nephelometry. The results obtained by nephelometry were compared with TCA-Ponceau S spectrophotometric method. Assays using BKC as precipitating reagent showed good precision and closest agreement with the TCA-Ponceau S dye binding method with a correlation coefficient of >0.98. Moreover, BKC showed satisfactory albumin-globulin response and analytical range; used less expensive and accessible chemicals; had automated pipetting and rapid measurement with an incubation time of 6min in their study.[7]

The turbidimetric 3% TCA method is commonly used manual method because it is simple to perform, rapid, familiar to many clinical laboratories but was found to have poor precision, repeatability and reproducibility; limited linearity and also required a larger sample volume. Benzalkonium Chloride (BKC) is a widely used disinfectant and is much less expensive than the other reagents, especially the dyes Coomassie Brilliant Blue and Pyrogallol Red Molybdate. It also inhibits bacterial contamination and this can be a reason for the better reagent stability.

Repeatability of measurements refers to the variation in repeat measurements made on the same subject under identical conditions. The mean CV for repeatability of BKC was better than TCA.TCA values were spread over a wider range as compared to BKC which can be seen in Figure 5. Hence, it was observed that BKC has better repeatability than 3% TCA.

Figure 5: Comparison of mean and range of absorbance values for repeatability studies by BKC and TCA methods (6mg/dl – 300mg/dl).



For reliability, 100% of the variability in measurement of absorbance for BKC and 99% for TCA were estimated to be due to genuine differences. One percent of differences for TCA were because of measurement errors. The within-subjects CV for BKC was better than TCA. Thus, BKC has proved to be a more reliable method than 3%TCA. Fatma Meric Yilmaz et al compared BKC with BTC, CBB and PYR and observed that the BKC method had lowest within and between-run CVs. In between-run imprecision studies, the BKC method had a significantly lower CV than the BTC method and this was thought to be related to reagent stability.[8] This finding is particularly important because the BTC and BKC methods are based on the same principle. The BKC method is also more advantageous than the BTC method in a manual procedure because the BKC method has a 10min incubation time at room temperature, whereas the BTC method requires incubation for 40–60min at room temperature.[5,15]

Reproducibility refers to the variation in measurements made on a subject under changing conditions. The changing conditions may be due to measurements being made by different observers or measurements being made over a period of time, within which the 'error free' level of the variable could undergo non-negligible change.[16] In the present study, the methods show greater variability as the protein concentration increases for which bias was calculated and it was observed that the difference in values was actual difference and not as a result of bias. TCA tends to give higher protein concentration.

Reagents required for one test by BKC method costs 6 Paisa while TCA method costs 13 Paisa for one test. Both the methods are cost effective but BKC method costs even less than TCA method. BKC requires half the sample volume as compared to TCA. As CSF is a precious sample this should also be taken into consideration.

A study done by Boer K et al found that CSF samples with spiked

amount of hemoglobin leads to overestimation of protein concentration when assayed by TCA. Using BTC which is similar to Benzalkonium chloride, measurement of protein concentration was minimally disturbed.[17] In the present study similar findings were observed.

In this study the reactivity to different proteins (albumin, gamma globulin and transferrin) was not evaluated separately by BKC method. It is suggested to measure response to albumin, gamma globulin and transferrin concentrations by BKC method for complete evaluation of this methodology.

CONCLUSION

BKC manual method is quick, simple, cost effective, requires less sample volume, has good precision and wide analytical range. It had agreed most closely with the TCA manual method in the present study. In imprecision studies, the reliability, repeatability and reproducibility of BKC method was better than TCA. Thus, BKC manual method can be recommended as an alternative method to TCA manual method for CSF total protein estimation.

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