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A Comparative Analytical Quality Evaluation Study between Two Methods for Blood Urea Nitrogen Estimation

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Abstract:*The present study was undertaken to compare the analytical quality of 1) Diacetyl Monoxime Method and 2) Berthelot's Method of estimation of blood urea. The performance characteristics were experimentally analysed to evaluate the analytical range, percentage recovery, sensitivity, accuracy, precision and detection limit of the two test methods. Performance of the methods were judged using statistical techniques. In a rural locality, due to interrupted electric power supply and inadequate UPS system for backup power, it was felt necessary to introduce a reliable manual backup method for blood urea test.Comparative analysis based on performance characteristics criteria indicate which of the two methods is acceptable. In the present study, Diacetyl monoxime method shows better analytical recovery, analytical sensitivity, detection limit with good accuracy and precision, as compared to Berthelot's method which is not acceptable.Although Diacetyl Monoxime method shows variation from that of CLIAstandards, it can be considered for used as a manual backup method, by stringent observance of specifiedstandard precautions during the analytical process, to minimize the errors and improve analytical quality. Good laboratory practices require individual laboratories to evaluate analytical quality of methods and apply routinely.*

Keywords:*quality control, performance characteristics, accuracy, precision, systematic error, random error*

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I. Introduction

Estimation of Blood Urea Nitrogen (BUN) forms one of the important tests for assessment of renal function. Estimation of any biochemical test in the clinical laboratory calls for accuracy, precision and reliability –an important aspect of analytical quality. The term "analytical quality" can be defined in several ways depending on the context it is referred. In terms of clinical laboratory standards, it means quality in performance characteristics, the accuracy of the method used for establishment and use of reference intervals, its long-term stability and its application on variousinstruments platforms.

Performance Parameters [1]

Various properties related to performance of the method are called performance parameters. Experimental assessment of quantitative results of each method and their objective evaluation is required to judge its acceptability. The acceptability of the method can be determined by comparing these results with the predetermined goals or performance standards. The following are the performance parameters of a method that needs to be evaluated:

Analytical Range:

It is the range of concentration of the analyte in the sample over which the method is applicable without modification. It is tested by linearity experiment in which reference solution containing a wide range of specific quantities of an analyte are determined by the candidate method. Ideally the calibration curve (the plot of response versus analyte concentration) should be linear and pass through the origin. Once the analytical range of the method has been validated it is termed as the reportable range of the method.

Analytical Recovery:

This refers to the ability of an analytical method to measure an analyte correctly when a known amount of it is added to authentic sample.

Analytical Sensitivity:

The analytical sensitivity of the method is the slope of the calibration curve. It is the ability of an analytical procedure to produce a change in the signal for defined change in the quantity.

Accuracy:

It is the closeness of the agreement between the measured value of an analyte and its true value. Systematic error is a measure of accuracy. In practice Accuracy or Systematic error is determined by 1) Comparison of observed value that is obtained by the candidate method to the true value obtained by several reference methodological techniques, and 2) Estimation of Systematic error which is comprised of two subdivisions

a) Constant Error: The magnitude of error is the same at different concentrations of the analyte. b) Proportional error: The magnitude of error is the percentage of concentration of analyte.

Precision:

The ability of an analytical method to produce the same value for replicate measurements of the same sample is known as precision. Random error is a measure of precision. Precision has three different components. a) Within run precision: This determines any imprecision in the run. b) Between run precision: This determines imprecision between runs on the same day. c) Between day precisions: This determines any imprecision on the different days.

Precision is usually estimated by a replication experiment in which the same sample is analyzed a minimum of 20 times and the standard deviation is calculated and compared with the desirable SD.

Information on the various components of error (Systematic Error or Random Error) can identify the source of errors and reduce their magnitude. On the other hand, what must be considered in judging the method is the overall effect of the components of error. i.e. Total Error. Total Error demonstrates how large the errors can be when random and systematic components occur in the same direction. Thus, total Error is one, which determines what analytical quality is achievable, and the ultimate acceptability of the method for its intended clinical applications.

Detection Limit:

It is the smallest concentration or quantity of an analyte (C_L),derived from the smallest measure $X_L$that can be detected with reasonable certainty for a given analytical procedure. The detection limit or Limit of Detection (LOD) depends on the amplitude of the blank readings and is also related to the precision of these measurements. The value of X_L is given by the equation:

$X_L = \underline{X}bl + kS_{bl}$where

$\underline{X}_{bl}$=Mean of Blank Measurements

S_{bl} = Standard Deviation of Blank Measurements

The values of $\underline{X}_{bl}$ and S_{bl} are found experimentally by making minimum 20 measurements

k is a coefficient which is determined by necessary confidence limits.

3 for 99 % Confidence limit

2 for 95 % Confidence Limit.

It is common for the terms analytical sensitivity and detection limit to be confused because the terms are interrelated – both are considered attributes of a sensitive method. In practice, an ideal method should have a high level of analytical sensitivity and low detection limit.

Analytical Specificity:

This term is related to accuracy and refers to the ability of an analytical method to determine exclusively the analyte it claims to measure without reacting with other related substances.

Measurement of Urea Nitrogen concentration in blood is useful in evaluation of renal function[2]. The function of every Clinical Chemistry laboratory is to perform qualitative and quantitative tests following standard procedures for maintaining accuracy and precision for proper diagnosis and treatment. The pressure for both cost reduction and quality improvement requires adopting better performing methods as a part of existing quality management program. Usually the enzymatic method of Urea estimation is considered the standard acceptable method, even for routine testing on fully automated biochemistry analysers. Our hospital is in a rural locality inMaharashtra State. Due to interrupted electric power supplythe machines get frequently malfunctioned requiring constant calibration, despite using UPS system for backup power.Moreover,in rural areas, technicalsupport is always delayed. Hence it was felt necessary to introduce and schedule a reliable manual backup method for the BUN test, especially when reports are required immediately for treating patients in emergency.

Thus, the main aim of the present study was to objectively evaluate the analytical performance of two methods viz. Diacetylmonoxime method and Berthelot's method of urea estimation, and compare the analytical performance of each method with enzymatic Kit method. Performance of these two methods, was evaluated by determination of linearityrange, analytical sensitivity, accuracy,precision anddetection limit. Thus, comparative analysis based on performance characteristics criteria should indicate which of the two method is acceptable as a

backup manual method for BUN estimation.Such method evaluation data can be found and easily assessed in open and commercial literature[3-10]. But good laboratory practices would require individual laboratories to use them only as a beginning part of reference and generate and apply routinely[11].

II. Methods

The present study was undertaken to compare the analytical quality of the two methods of Urea estimation. i.e. Diacetyl Monoxime Method (DAM)[12], and Berthelot's Method.[13].DAM method utilizes the Fearon reaction, wherein urea condenses with diacetyl monoxime to form diazine derivative under acidic conditions to form a pink colored complex in the presenceof ferric ions and thiosemicarbazide; which is measured spectrophotometrically at 530 nm. In Berthelot's method, urease enzymes act on urea to give carbon dioxide and ammonia. Ammonia liberated reacts with base reagent to give chloramine which in turn reacts with phenol from color reagent to form blue colored complex, which ismeasured at 620 nm. In the enzymatic kit method, urea is hydrolyzed by urease enzyme to form Carbon dioxide and Ammonia. Ammonia produced reacts with α-ketoglutarate to obtain Glutamate in the presence of glutamate dehydrogenase (GLDH). In the process NADH gets oxidized to NAD^+ which is measured as decrease in absorbance at 340 nm. The performance characteristics were determined by experimental analysis of the following: -

1. Determine linearity range. i.e. the range of concentrations of analyte over which the method is applicable.
2. Find the percentage recovery of the analyte in the unknown specimen by recovery experiment.
3. Measure Analytical Sensitivity of both the methods using calibration curve.
4. Study accuracy by comparison of experiment results of each candidate method with enzymatic kit method.
5. Study Precision of both methods by replicate measurement experiment.
6. Determine Detection Limit of the two methods to reveal lowest quantity of the analyte that could be tested.

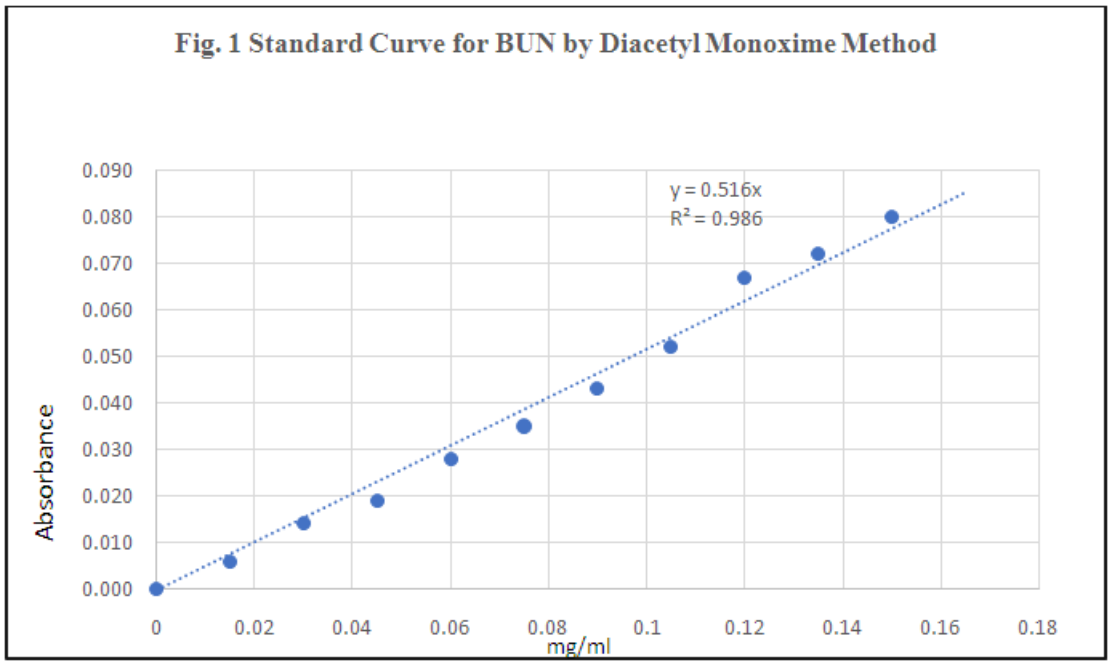
These parameters were evaluated using various statistical analysis to compare, judge and determine the performance and acceptability of the two methods.

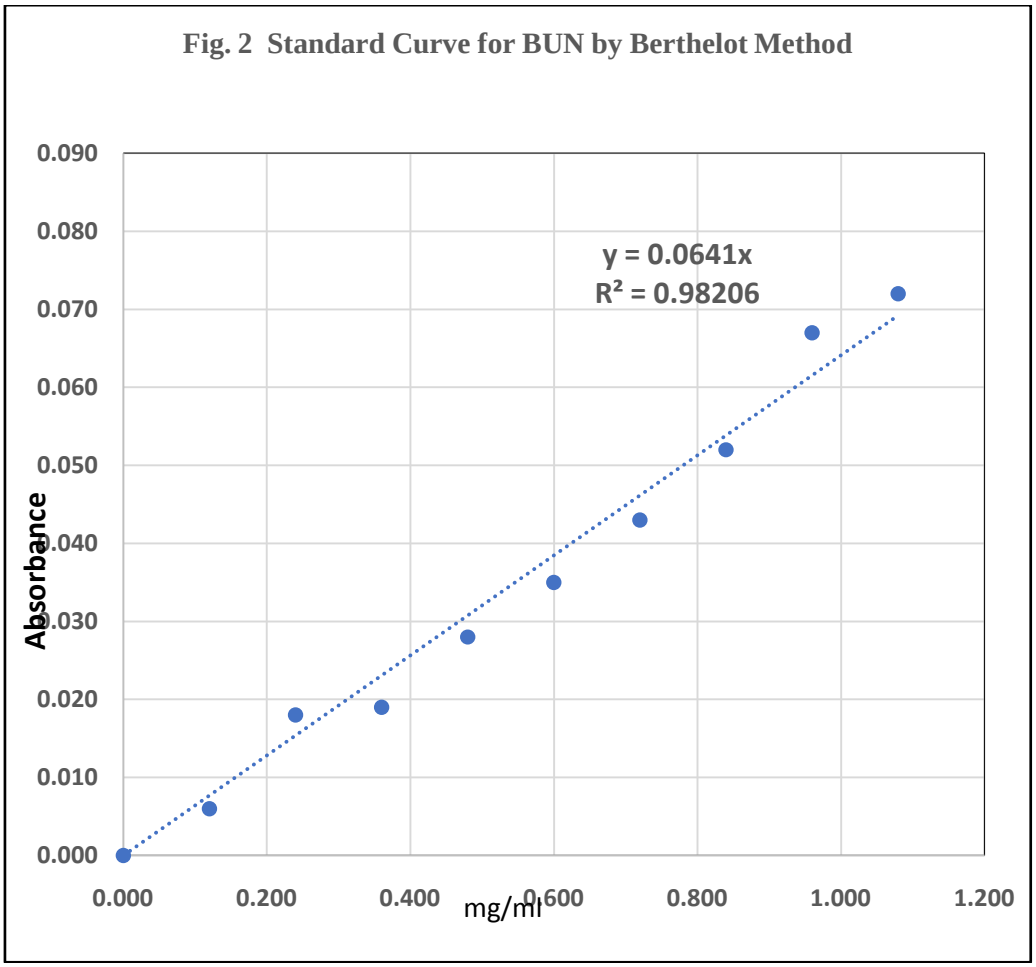
III. Results

1. Analytical Range:

Diacetyl Monoxime Method: Fig. 1 shows plot of BUN concentration in mg/ml v/s absorbance obtained at 530 nm. The figure shows calibration curve that is a straight line passing through the origin. The curve is found linear up to 0.105 mg/ml.

Berthelot's Method: Fig. 2 shows plot of BUN concentration in mg/ml v/s absorbance obtained at 620 nm. The figure shows calibration curve that is a straight line passing through the origin. The curve is found linear up to 0.90 mg/ml.





2. Analytical Recovery:

This was determined by performing a "recovery" experiment. The results obtained are depicted in Table 1 & Table 2

Table 1:Recovery of BUN by Diacetyl Monoxime Method

Sample	ml. of pooled specimen (1:10)	ml.of BUN solution added (100 mg/dl)	Conc. of BUN, in Pooled Specimen (mg/dl) (a)	Conc. of added BUN (mg/dl) (b)	BUN Recovered (mg/dl) (c)	Percentage Recovery (c/b x100) (d)	Percentage Proportional Error 100 - d
1	0.25	0.75	17.45	75	69.975	93.30	6.70
2	0.50	0.50	18.29	50	46.500	93.00	7.00
3	0.75	0.25	19.67	25	22.988	91.95	8.05

Table 2: Recovery of BUN by Berthelot'sMethod

Sample	ml. of pooled specimen (1:10)	ml.of BUN solution added (100 mg/dl)	Conc. of BUN, in Pooled Specimen mg/dl (a)	Conc. of added BUN (mg/dl) (b)	BUN Recovered (mg/dl) (c)	Percentage Recovery (c/b x100) (d)	Percentage Proportional Error 100 - d
1	0.25	0.75	18.48	75	66.6375	88.85	11.15
2	0.50	0.50	19.65	50	42.6250	85.25	14.75
3	0.75	0.25	20.32	25	20.5825	82.33	17.67

The amount of BUN recovered was determined by subtracting the amount of BUN originally present in the respective pools from the amount measured or found. Percent recovery was then calculated by dividing the amount of BUN recovered by the amount of BUN added and multiplied by 100.

3. Analytical Sensitivity:

The sensitivity of the method was determined from the slope of the calibration curve as shown in Fig. 1 slope y/x = 0.52for Diacetyl Monoxime Method; and in Fig 2 slope y/x = 0.06for Berthelot Method.

4. Accuracy of the method

Accuracy of the method was determined by the Systematic Error estimated by the "Comparison of method experiment". Candidate method was compared with Enzymatic kit method. (Table 3, Table 4 and Fig. 3, Fig 4)

Table 3: Accuracy of BUN estimation by comparison of the Diacetyl Monoxime Methodwith Kit Method

Parameters	Formula	Value of DAMO method v/s kit method
Correlation Coefficient (R^2)	$\frac{[\Sigma XY - (\Sigma X \Sigma Y)/n]}{\sqrt{[\Sigma X^2 - (\Sigma X)^2/n]\,[\Sigma Y^2 - (\Sigma Y)^2/n]}}$	0.989
Slope (b)	$\frac{[\,\Sigma XY - (\Sigma X \Sigma Y)/n]}{[\,\Sigma X^2 - (\Sigma X)^2/n]}$	1.006
Y – Intercept (a)	$\bar{y} - (b)\bar{x}$	1.817
Proportional Error %	[b – 1] x 100	0.605
Constant Error at 5 mg/dl (Y-Intercept)	$\bar{y} - (b)\bar{x}$	1.817
Systematic Error at 5 mg/dl	[Yc – Xc]	1.847
Total Error at 5 mg/dl [Systematic Error + Random Error]	[Yc – Xc] + S_{obs} x 4	4.847

X= Individual observation on X-axis
Y= Individual Observation on Y-axis.
Xc= Value of X taken at decision level, here 5 mg/dl
Yc=Value of Y taken at decision level, here 5 mg/dl.
S_{obs}= SD of differences between test & reference method values

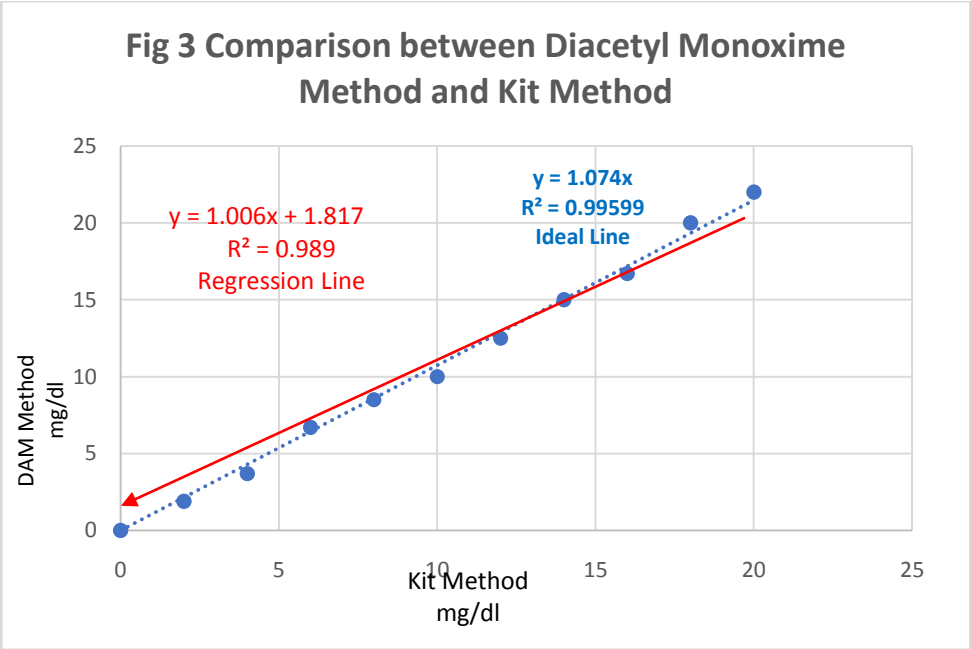
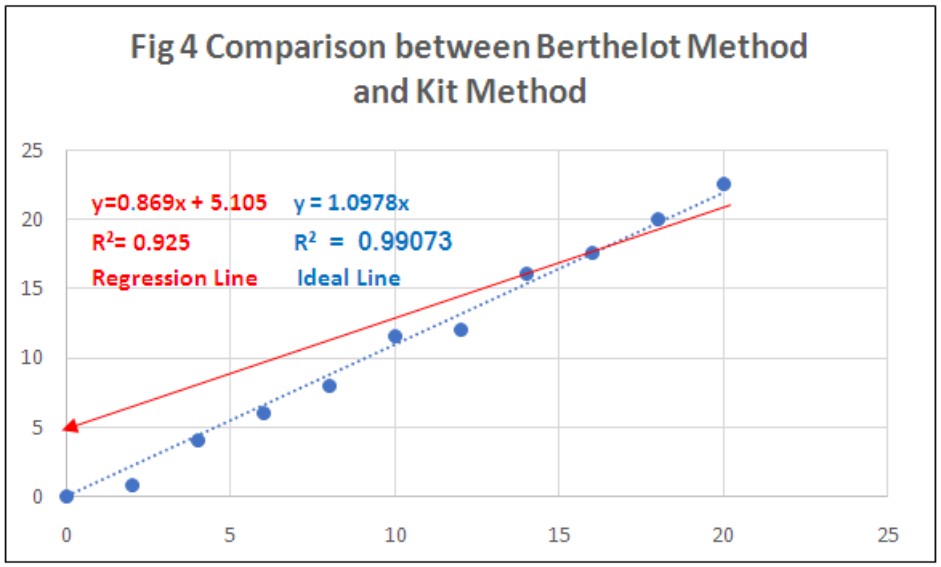


Table 4: Accuracy of BUN estimation by comparison of the Berthelot Method with Kit Method

Parameters	Formula	Value of Berthelot's method v/s kit method
Correlation Coefficient (R^2)	$\frac{[\Sigma XY - (\Sigma X \Sigma Y)/n]}{\sqrt{[\Sigma X^2 - (\Sigma X)^2/n]\,[\Sigma Y^2 - (\Sigma Y)^2/n]}}$	0.925
Slope (b)	$\frac{[\Sigma XY - (\Sigma X \Sigma Y)/n]}{[\Sigma X^2 - (\Sigma X)^2/n]}$	0.8697
Y – Intercept (a)	$\bar{y} - (b)\,\bar{x}$	5.1057
Proportional Error %	[b - 1] x 100	- 13.0314
Constant Error at 5 mg/dl (Y-Intercept)	$\bar{y} - (b)\,\bar{x}$	5.1057
Systematic Error at 5 mg/dl	[Yc – Xc]	4.45
Total Error at 5 mg/dl [Systematic Error + Random Error]	$[Yc - Xc] + S_{obs} \times 4$	7.85

X= Individual observation on X-axis
Y= Individual Observation on Y-axis.

Xc= Value of X taken at decision level, here 5 mg/dl
Yc= Value of Y taken at decision level, here 5 mg/dl.
S_{obs}= SD of differences between test and reference method values



5. Precision:
Table 5 and Table 6 shows the Mean, Standard Deviation and coefficient of variation of the respective components of precision; viz. Within run precision, between run precision and between day precision.

Table No 5 : Components of Precision – Diacetyl Monoxime Method

Component of Precision	Mean (mg/dl)	SD (mg/dl)	CV (mg/dl)
Within Run Precision	19.370	0.7391	4.261
Between Run Precision	19.473	1.6282	8.361
Between Day Precision	19.414	1.6867	8.688

Table No 6: Components of Precision – Berthelot's Method

Component of Precision	Mean (mg/dl)	SD (mg/dl)	CV (mg/dl)
Within Run Precision	20.362	1.4550	7.148
Between Run Precision	20.414	1.5196	7.444
Between Day Precision	20.567	1.5635	7.601

6. Detection Limit:
The detection limit was calculated using the following formula,

Detection Limit = $X_L = X_{bl} + k\ S_{bl}$
Where X_{bl}= Mean of Blank Measurements
S_{bl} = SD of Blank Measurements
K = 2 for 95% Confidence Limit.

Diacetyl Monoxime Method:
Detection Limit = 0.0530 mg/dl.

Berthelot's Method:
Detection Limit = 0.226 mg/dl.

IV. Discussion

Several analytical methods are available for estimating the analyte of interest and adopting a method for routine use in Clinical Chemistry Laboratory. This process entails proper selection and evaluation using rigorous quality guidelines.Selecting a method involves review of relevant scientific literature, sample collection, storage, transport, volume of sample required for the assay, long term stability of the reagents and reference materials required, cost of the operation of the assay,hazards, waste disposal and availability of instrument and technical support.

The present study deals with an objective method of evaluation of the two methods – Diacetyl Monoxime method and Berthelot's method for the estimation of Blood Urea Nitrogen (BUN). The evaluation process involved quantitative assessment of its performance parameters like analytical range, analytical recovery, analytical sensitivity, accuracy, precision, detection limit and analytical specificity. The criterion for judging the acceptability of errors for estimation of BUN is shown in Table No. 7.

Table No 7: Criteria for judging the acceptability of the two methods

TYPE OF ERROR	EXPERIMENT	CRITERIA
Random Error	Replication	$4 \times S_{obs < 100 mg/dl}$
Proportional Error	Recovery	$[(R-100/100)]5_{< 100 mg/dl}$
Constant Error Systematic Error	Comparison of Methods	$[(a+5b) -5]_{< 100 mg/dl}$
Total Error	Replication and Comparison	$4 \times S_{obs} + [(a+5b) -5]_{< 100 mg/dl}$

Where: S_{obs}= SD determined in replication experiment
R = Average recovery % determined in a recovery experiment
a = Y intercept of the regression line
b = slope of regression line

Analytical Range:

The calibration curve or linearity plot is a straight line passing through the origin. The curve shows excellent linearity up to 0.105 mg/ml for DAM method, (Fig No 1) and 0.90 mg/ml for Berthelot's method(Fig. No 2). This is the analytical range over which the method is applicable without any modification.

Analytical Recovery:

In the DAM method, the average recovery obtained is 92.75 %, which accounts for 7.25 % proportional error (Table No 1). This is more than the allowable total error 2.4 % as per CLIA recommendation.[1]This error may be accounted for taking the readings of hot solution which is cooled to room temperature, during which the glass cuvette gets expanded due to heat. In the Berthelot's method, the average recovery accounted for 85.47 % which accounts for 14.523 % proportional error (Table No 2).This is more than the allowable total error of 2.4 % as per CLIA recommendation. This error may be attributed to delay in recording the readings for the blue colored complex or incubating the specimen for longer period.

Analytical Sensitivity:

The sensitivity obtained is 0.52 (DAM method) and 0.06 (Berthelot's method) obtained from slope of calibration curve in Fig no. 1 and Fig. No. 2. The value of the slope signifies change in absorbance for a unit change in concentration.

Accuracy:

Regression analysisgives the following statistical data (DAM method)
Slope = 1.006, Y intercept = 1.817and correlation coefficient r = 0.9895.

The slope is close to 1.00, showing that proportional error is 0.6 %. The Y intercept is close to zero indicating constant error of 1.817. High value of r = 0.9895, confirms that simple linear regression should be satisfactory for analyzing data. To judge the acceptability of this method, systematic errors must be estimated at decision levels of interest.The systematic error at decision level of 5 mg/dl is + 1.847. Yc=a+bx5, Yc=1.817 + 1.006(5.0) = 6.847. Also note that the constant and proportional components are not in opposite direction and their effects are not counter balanced. Although the systematic error stand alone is small, the total error (TE) of 4.847 is more than the specified allowable total error (TEa = 2.4 mg/dl); where TE = SE + RE. RE is 4 times the day to day SD.TE estimated at 5 mg/dl is found to be 4.847 [1.847 + 4 (0.75)].
Regression analysis gives the following statistical data (Berthelot's method)
Slope = 0.8697, Y intercept = 5.1057 and correlation coefficient r = 0.925.
These statistics are interpreted as follows:

The slope is close to 1.00, showing that proportional error is – 13.03 %. The Y intercept is higher, indicating a constant error of 5.1057 mg/dl. The high value of r = 0.925confirms satisfactory outcome of simple linear regression for analyzing data. However, its acceptability can be judged by evaluation of systematic errors at the decision levels of interest.The systematic error at the decision level of 5 mg/dl is + 4.45 mg/dl.
Yc=a+bx5, Yc=5.1057 + 0.8697(5.0) = 9.452. Constant component (5.1057) and
proportional component (- 13.03) are in opposite direction, their effects being partially counterbalanced. The systematic error (+4.45) is by itself large and more than the specified allowable total error (TEa of 2.4 mg/dl).
TE = SE + RE. RE is 4 times the day to day SD.TE estimated at 5 mg/dl is found to be 7.85 [4.45 + 4 (0.85)].

Precision:

Analysis of data - DAM method: -

The precision or the ability of an analytical method to produce the same value for replicate measurements of the same sample was determined from 3 components viz. Within run precision, Between run precision, and between day precision. These components as determined by replicate measurements were estimated by calculating its respective mean, standard deviation and coefficient of variation. The values depicted for SD in Table No. 5 nearly confirm to the set levels of 0.6 mg/dl in respect of within run precision. However, the values obtained for between run (1.6282 mg/dl) and between day (1.6867 mg/dl) varies grossly from 0.6 mg/dl. This infers that the method does not produce similar results on use of different glassware and reagents. The observed CV values for between run and between day are also not in the acceptable range of the allowable CV value of 4 mg/dl[1](Table No. 5).

Analysis of data – Berthelot's method: -

The values as shown in Table No 6 in respect of within run (1.45 mg/dl), between run (1.52 mg/dl) and between day (1.56 mg/dl) does not confirm to the set levels of 0.6 mg/dl. This indicates dissimilarities of results on use of different glassware, reagents and time. Moreover, the CV values (Table No 6) are also not in the acceptable range of the allowable value of 4 mg/dl [1]

Detection Limit:

The detection limit observed for Dam method is 5.30 x 10^{-2}mg/dl and for Berthelot's method it was observed to be 22.6 x 10^{-2}mg/dl

Comparison between the two methods of analysis:

Table No 8 depicts the comparison of the performance parametersbetween Diacetyl monoxime method and Berthelot's method.

Table No 8: Comparison of performance

Performance Parameters	Diacetyl Monoxime Method	Berthelot's Method
Analytical Range	up to 0.105 mg/ml.	up to 0.90 mg/ml.
Analytical Recovery	92.65 %	85.47 %
Analytical Sensitivity	0.52	0.06
Accuracy [Systematic Error]	1.847	4.45
Total Error	4.847	7.85
Precision [Average SD]	0.75 mg/dl	0.85 mg/dl
Detection Limit	0.053 mg/dl	0.226 mg/dl

V. Conclusion

All in all, the performance characteristics of the Diacetyl monoxime method shows better analytical recovery, analytical sensitivity, detection limit with good accuracy and precision than Berthelot's method; when each test method is compared with the enzymatic kit method.The analytical range of the Berthelot's method (0.90 mg/dl) scored over DAM method (0.105 mg/dl). However, the Diacetyl Monoxime method shows variation from that of CLIA standards, hence not entirely reliable. It can be used as a manual backup method for the BUN test by taking certain precautions like use of same apparatus, appropriate time of taking readings, maintaining water bath temperature and timing, cooling the solution to room temperature before recording the readings.

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