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A comparative study on the estimation of serum Creatinine levels by Jaffe's and Enzymatic methods at different levels of serum Bilirubin

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Abstract

In the present study, based upon their total serum bilirubin values ranging from 0.3-30.8 mg/dl, 47 patients visiting OPD and IPD of HIMS were divided into five groups. Their serum creatinine levels were estimated by the Jaffe's and the Enzymatic methods. Total serum bilirubin concentration was estimated by the timed endpoint Diazo method. Results obtained during the studies revealed that in all the five groups, estimation of serum creatinine by the Jaffe's method always gave significantly higher values ($p< 0.01$) as compared to their estimated values by the Enzymatic method at the identical serum bilirubin concentrations. The differences in the mean values of serum creatinine obtained between the two methods were found to significantly increase with the increase in the serum total bilirubin concentrations. At serum total bilirubin concentration of < 2.0 and >20.0 mg/dl, the mean differences between the Jaffe's and Enzymatic methods were found to be 0.2206 ± 0.0508 and 0.6786 ± 0.0530 mg/dl respectively, resulting in an approximately three-fold increase in the difference in the estimated serum creatinine concentration by the two methods. The highly significant enhancement in the differences in the estimated values of serum creatinine, between the Jaffe's and the Enzymatic method, with an increase in serum bilirubin concentrations, are attributed to the negative and positive interference in creatinine estimation caused by lower and higher concentrations of total serum bilirubin respectively.

Keywords: Creatinine, Bilirubin, Jaffe's method, Enzymatic Method

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Introduction

The best indicator of kidney function is considered to be the Glomerular Filtration Rate (GFR). Chronic kidney disease is defined as the one having a GFR <60ml/min/1.73m^2 for three months or more irrespective of the cause.[1] GFR measurement procedures employing either exogenous markers, such as Inulin, Iohexol, Iothalamate and Cr^{51}-EDTA or endogenous substances, such as urea and creatinine, are not suited to be routinely used for detecting kidney ailments. These procedures are not only very cumbersome and time consuming but also very expensive. Because of the inverse relationship between serum creatinine and GFR, efforts have been directed at more convenient urine free estimation of GFR, where serum creatinine estimation assumes clinical significance.[2]

In 1886, Jaffe evolved an assay system for routine estimation of creatinine in serum and urine samples.[3] With the advent of automation especially after 1957, Jaffe's assay system has undergone various modifications. Despite the known interference by various small molecular weight substances such as glucose, pyruvate, acetoacetate, bilirubin and cefoxitin etc in estimation of serum creatinine using alkaline picrate, Jaffe's method is still being routinely used in clinical biochemistry laboratories.[4-7] The presence of glucose and bilirubin in test samples cause underestimation of creatinine by the Jaffe's method due to the inhibition of the complex formation between creatinine and alkaline picrate. Glucose is known to slowly reduce the conversion of picrate to picramate.[8] Bilirubin on the other hand has been shown to be oxidized to biliverdin under alkaline conditions resulting in the decrease in absorption at 520 nm.[9] In contrast, reaction between acetoacetate, ascorbic acid or cefoxitin (a first generation cephalosporin) with picrate result in formation of a complex having absorbance at 520 nm resulting into the positive interference in creatinine estimation. As compared to creatinine, acetoacetate, having more affinity for picrate reacts very fast to cause positive interference. Foetal haemoglobin (HbF) also causes interference which often is being overlooked. In contrast to adult heamoglobin which is known to immediately turns brown under alkaline conditions, HbF being alkali resistant inhibits the reaction between creatinine and alkaline picrate resulting in under estimation of creatinine by the Jaffe's method. To overcome the above mentioned interferences in creatinine estimation by non enzymetic Jaffe's method, Boyne and his colleagues developed a highly specific Enzymatic method.[10] Enzymatic method employs three enzymes, Creatininase (EC 3.5.2.10), Creatinase (EC 3.5.3.3) and Sarcosine oxidase (EC 1.5.3.1) which in a step-wise method converts creatinine $\rightarrow$creatine $\rightarrow$sarcosine$\rightarrow$ glycine + HCHO + H_2O_2. Using a Leuco dye, Peroxidase enzyme is employed to spectrophotometrically measure the concentration of H_2O_2 produced. Although, the interference by glucose, pyruvate, acetoacetate and cefoxitin gets eliminated in the Enzymatic method, depending upon the concentrations of both bilirubin and creatinine, bilirubin

is known to cause negative interference in creatinine estimation by this method.

The objective of the present study was to conduct a comparative study on the estimation of serum creatinine levels by Jaffe's and Enzymatic methods at different levels of serum bilirubin.

The present study assumes clinical significance in the management of kidney ailments.

Materials and Method

After obtaining the approval from the Ethical and Research Committee of Himalayan Institute of Medical Sciences, Dehradun, Uttrakhand, the present study was carried out in the Biochemistry department of the institute. Informed consent was taken from the patients. A total 47 OPD and IPD subjects were selected for the present study. Serum total bilirubin and creatinine estimations were done by the following methods.

Creatinine Estimation: Enzymatic and Jaffe's methods were employed for the estimation of creatinine in the various serum samples obtained from 47 patients.

1. **Jaffe's Method:** The concentration of creatinine in the samples was determined by the rate modified Jaffe's method[3] using Beckman Coulter, Synchron DXC System.

Principle: In alkaline conditions, creatinine reacts with picrate to form creatinine-picrate complex having significantly increased absorbance at 520 nm as compared to the actual chromogen, picrate. This increase in absorbance at 520nm is directly proportional to the serum creatinine concentration.

The ratio of 1:11 between the sample and the reagent is automatically maintained in the SYNCHRON DXC system.

Reaction Scheme:

Creatinine + Picric acid $\xrightarrow{\text{Alkaline medium}}$ Creatinine picrate (Red coloured complex)

Reagents Used:

Picric Acid	8.1 mmol/L
Buffered to pH with NaOH	> 13.3

Also non-reactive chemicals necessary for optimal system performance.

Reagent preparation: 1 drop of Antifoam reagent is added to compartment A of reagent cartridge and mixed gently.

Linearity: The method obeys Beer's law up to 25.0 mg/dl of creatinine in serum and for higher concentrations the samples were suitably diluted with saline and reanalyzed. The appropriate dilution factor was applied while calculating the final results.

2. **Enzymatic Method:** Ortho-Clinical Diagnostics, VITROS 250 Chemistry System based upon the Enzymatic method was used to estimate the concentration of creatinine in the various samples.

Principle: The VITROS system uses a creatinine slide which is a multilayered analytical element coated on a polyester support. The spreading layer on the underlying layers uniformly distributes the drop of the applied sample. In a rate determining step the creatinine diffused in the reagent layer gets hydrolysed by creatininase (creatinineamidohydrolase) to form creatine. The creatine thus produced gets converted to sarcosine and urea by creatinase (creatineamidohydrolase). Sarcosine oxidase converts sarcosine to glycine, formaldehyde and hydrogen peroxide. In the final reaction a coloured product gets produced by the oxidation of Leuco dye by the peroxidase enzyme, The slide is incubated after the addition of the sample resulting in the oxidation of endogenous creatine. The concentration of creatine present in the sample is proportional to the difference in reflection density (reflectance) measured at 670nm at 2 specific time points.[10]

Reactions Scheme:

Creatinine + H_2O $\xrightarrow{\text{Creatininase}}$ Creatine

Creatinine + H_2O $\xrightarrow{\text{Creatinase}}$ Sarcosine + Urea

Sarcosine + O_2 + H_2O $\xrightarrow{\text{Sarcosine Oxidase}}$ Glycine + Formaldehyde + H_2O_2

H_2O_2 + Leuco dye $\xrightarrow{\text{Peroxidase}}$ Dye + $2H_2O$

Reagents:

Slide Ingredients

Reactive ingredients per cm^2

Creatinineamidohydrolase 0.20 U

Creatineamidohydrolase 4.7 U

Sarcosine oxidase 0.55 U

Peroxidase 1.6 U

2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis(dimethylaminophenyl)imidazole (leuco dye) 32 µg

Reagent preparation: No reagent preparation is required for the slides used for serum creatinine estimation on the dry chemistry analyser.

Linearity: The dynamic range for serum creatinine is from 0.05- 14.0 mg/dl and for higher concentrations the samples were diluted using VITROS 7% BSA. The appropriate dilution factor was applied during calculations.

Total Bilirubin Estimation: The total bilirubin concentration in the samples was determined by the timed endpoint diazo method[11] using beckman coulter, synchron dxc system.

Principle: Azobilirubin having absorption maxima at 520 nm is formed when bilirubin reacts with diazo reagent in the presence of caffeine, benzoate and acetate as accelerators. This increase absorbance at 520nm is directly proportional to the total bilirubin concentration of the samples.

Reaction Scheme:

Total Bilirubin + Diazo + H^+ $\xrightarrow{\text{Caffeine, Benzoate, Acetate}}$ Azobilirubin (blue color)

The SYNCHRON DXC System automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio used is one part sample to 35 parts of the reagent.

Reagents:

Sodium Benzoate	347 mmol/L
Caffeine	173.9 mmol/L
Sulfanilic acid	27 mmol/L
HCl	50 mmol/L
Sodium Nitrite	0.36 mmol/L
Sodium Acetate	609 mmol/L

Plus non-reactive chemicals necessary for optimal system performance.

Reagent preparation: Quantitatively 100 microliters (0.1 mL) of the contents from the smallest compartment (C) are transferred into the centre compartment (B).

The cartridge is gently inverted several times to ensure adequate mixing. 1 drop of Antifoam reagent is added to the compartment A of reagent cartridge and mixed gently.

Linearity: The method obeys Beer's law up to 30.0 mg/dl in serum and for higher concentrations the samples were suitably diluted and reanalyzed. The appropriate dilution factor was applied while calculating the final results.

At least two levels of Biorad quality controls were analyzed daily for each of the above three methods as per NABL norms.

Statistical Analysis: For statistical analysis SPSS version 17.0 was employed. ANOVA was applied to determine the statistical significance of the results obtained.

Results

Based upon their serum total bilirubin concentrations, 47 patients were divided into five groups. Patients in group I, II, III, IV and V had mean serum total bilirubin concentrations of <2.0, 2.1-5.0. 5.1-10.0, 10.1-20.0 and >20.0 mg/dl, respectively. The study presented in table demonstrate that in all the groups, as compared to the Enzymatic method, Jaffe's method always gave higher values of serum creatinine at the identical bilirubin concentrations. For group I, II, III, IV and V, the mean values of serum creatinine estimated by Jaffe's method were 1.012 ± 0.145, 1.2377±0.177, 1.378±0.115, 1.432±0.088 and 1.504±0.105 mg/dl respectively. At the identical serum total bilirubin concentrations in the above groups, the mean serum creatinine values estimated by the Enzymatic method were estimated to be 0.781 ± 0.150, 0.868±0.178, 0.968±0.095, 0.959±0.095 and 0.826±0.097 mg/dl respectively. The mean differences between the Jaffe's and Enzymatic methods in I-V groups were found to be 0.221 ± 0.051, 0.368±0.063, 0.467±0.018, 0.468±0.016 and 0.678±0.053 mg/dl respectively. The overall mean difference in estimated creatinine levels by the Jaffe's and the Enzymatic methods was determined to be 0.388±0.163 mg/dl. All the above mean differences were found to be statistically significant ($p<0.01$).

Results presented in Fig. 1 further demonstrate that the difference in the estimated creatinine values between the two methods gets progressively increased with increase in the concentration of serum total bilirubin. With the increase in the serum total bilirubin concentrations from < 2.0 to > 20.0 mg/dl, the mean difference between the two methods was found to increase by approximately three folds.

Discussion

In the present study at serum total bilirubin concentrations ranging from 0.3 to 33.0 mg/dl, creatinine estimation by the Jaffe's method always gave significantly higher values as compared to the values determined by the Enzymatic method. The observed mean differences in creatinine values, determined by the two methods, were found out to be highly significant ($p<0.01$). The above differences in creatinine values between the two methods is due to the fact that the Enzymatic method has a distinct advantage over the Jaffe's method because it is not only highly specific but also the small molecular weight dialyzable substances such as pyruvate, aceto acetate, glucose etc. which are known to cause either positive or negative interference in creatinine estimation by the Jaffe's method, fail to influence the creatinine estimation by the Enzymatic method.[12] The non-specific interaction between the small molecular weight substances present in the serum samples with alkaline picrate leads to the over estimation of serum creatinine by the Jaffe's method.[13]

Although the Enzymatic method is relatively being more specific because it eliminates the interference by the small molecular weight substances, yet, it has its own limitations. Some substances present in serum particularly bilirubin has been reported to compete with assay substrates / acceptors for H_2O_2 produced during the reaction resulting in the interference of creatinine estimation by the Enzymatic method.[14] The problem gets further compounded in patients requiring liver transplant, where their serum bilirubin concentrations often exceed the recommended limits for bilirubin interference by the Jaffe's and Enzymatic methods. Interestingly, the present studies clearly indicate that although the serum total bilirubin concentrations present in different patients were not found to significantly influence the estimation of serum creatinine by either Jaffe's or the Enzymatic method, yet the mean differences observed between the two methods became highly significant at very high serum total bilirubin concentrations (> 20.0 mg/dl). This observation can possibly be explained by postulating that various molecules present in serum may have different mechanism of action to influence/ cause interference in the creatinine estimation by the Jaffe's and the Enzymatic methods.

Mechanism of interference by bilirubin in the estimation of Creatinine: Although bilirubin is known to cause negative interference in estimation of serum

creatinine by both the Jaffe's and the Enzymatic methods, however, its mechanism of causing interference in the two methods is quite different. In Jaffe's method, bilirubin gets converted to biliverdin under alkaline conditions. Biliverdin thus formed has λmax at 630 nm which significantly decreases the absorbance of the creatinine–picrate complex observed at 520 nm. In the Enzymatic method bilirubin directly competes with the chromogen dye for H_2O_2 resulting in negative interference.

The question that naturally arises is that if in both the methods bilirubin causes negative interference, then why the difference between the two methods increases very significantly at higher concentrations of bilirubin? Since, substrates and chromogen in the two methods react on mole to mole basis, each method has a specific upper limit for the substrate where it obeys Beer's Law. As the λmax of bilirubin absorbance (510 nm) almost coincides with that of creatinine-picrate complex ie 520 nm, hence, at higher concentrations of serum bilirubin, the concentration of either NaOH and/or picrate may become a limiting factor resulting into the positive interference by unreacted bilirubin in the Jaffe's method. Hence the negative and positive interference caused by lower and higher concentrations of total bilirubin respectively, in the creatinine estimation by the two methods, would result into significantly enhancing the difference between the two methods at higher concentration of bilirubin. With the increase in total bilirubin concentration from <2.0 to > 20.0 mg/dl, the mean difference in creatinine values between the two methods was found to approximately increase by three folds.

Since more than 50 substances have been shown to interfere in the serum creatinine estimation by the Jaffe's and/or the Enzymatic methods, the estimation of the true creatinine value, which is so vital for accessing the kidney status, posses a real problem to the biochemists. To determine the accuracy and do calibration of various methods, the study of the mechanism of action of various interfering substances assumes practical importance and the present study is an attempt in that direction.

Ethical Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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References

1. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification and stratification. Kidney Disease Outcome Quality Initiative. Am J Kidney Dis. 2002;39:S1–S246.
2. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron. 1976;16:31–41.
3. Jaffe M. On the precipitation which picric acid produces in normal urine, and on a new reaction of creatinine (translated form). Journal of physiological chemistry. 1886;10:391–400.http://vlp.mpiwg-Zerlin.mpg.de/references?id=lit16635&page=p0391.
4. Cook JG. Factors influencing the assay of creatinine. Ann Clin Biochem. 1975;12:219–32.
5. Bowers LD, Wong ET. Kinetic serum creatinine assays. II. A critical evaluation and review. Clin Chem. 1980;26:555–61.[PubMed]
6. Pardue HL, Bacon BL, Groeger Nevius M, Skoug JW. Kinetic study of the Jaffe reaction for quantifying creatinine in serum:1. Alkalinity controlled with NaOH. Clin Chem. 1987; 33:278–85.
7. Spencer K. Analytical reviews in clinical biochemistry: the estimation of creatinine. Ann Clin Biochem 1986;23:1-25.
8. Bowers LD, Wong ET. Kinetic creatinine assays. II. A critical evaluation and review. Clin Chem 1980;26:555-61.
9. Knapp ML, Hadid 0. Investigations into negative interference by jaundiced plasma in kinetic Jaffe methods for plasma creatinine determinations. Ann Clin Biochem 1987;24:85-97.
10. Boyne P, Robinson BA, Murphy P, McKay M. Enzymatic correction of interference in the kinetic Jaffe reaction for determining creatinine in plasma. Clin Chem. 1985;31:1564–5.
11. Malloy HT and Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. JBiolChem1937;119:481-90.
12. Gerard SK, Khayam-Bashi H. Characterization of creatinine error in ketotic patients. Am J Clin Pathol 1985;84:659-64.
13. Chaudhary SS, Shahand JP, Mahato RV. Interference of Bilirubin in Creatinine Value Measurement by Jaffe Kinetic Method. ACCLM 2015;1(1) 25-28.
14. Crocker H, Shephard MD, White GH. Evaluation of an enzymatic method for determining creatinine in plasma. J Clin Pathol. 1988;41:576–81.