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Approach to overcome the bilirubin interference in the estimation of serum creatinine by Jaffe's method

C.N. Shruthi[1], V. Karishma[2] and A.G. Thivyahprabha[3*]

[1]Department of Biochemistry, MVJ Medical College and Research Hospital, Dandupalya, 30th KM milestone, NH-4, Kolathur P.O, Hoskote-562114, Bengaluru Rural District, Karnataka, India, [2]Medical Student, MVJ Medical College and Research Hospital, Dandupalya, 30th KM milestone, NH-4, Kolathur P.O, Hoskote-562114, Bengaluru Rural District, Karnataka, India and [3]Department of Biochemistry, St. Peter's Medical College Hospital & Research Institute, Opposite to SIPCOT 2, Hosur-635109, Tamilnadu, India

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Abstract: *Introduction:* Bilirubin has negative interference on creatinine measurement by routinely used Jaffe's Kinetic method. Hence this study was conducted to find out a possible solution to the problem of bilirubin interference. Simple chemical method has been validated for serum creatinine estimation which is free from bilirubin interference. The performance of this method has been compared with the high performing enzymatic method. *Aim:* To estimate the serum creatinine in samples having high bilirubin level. *Materials and Methods:* 60 serum samples with bilirubin value>2 mg/dl were included in the study. Samples were divided into 3 groups each containing 20 samples, based on the bilirubin value. Each group was divided into two subgroups of 10 samples each. Initially serum creatinine was estimated by both enzymatic method and Jaffe's kinetic method. Later serum creatinine was measured once again in all the subgroups by both methods after the preincubation of sample with NaOH for 5 min and deproteinization with Trichloroacetic acid. The statistical analysis is done by t test. *Results:* There was a significant increase in the creatinine obtained by Jaffe's method after deproteinization and preincubation of the sample when compared to the creatinine obtained by Jaffe's method before preincubation and deproteinization. The creatinine estimated after the deproteinization and preincubation by Jaffe's method are similar to the creatinine obtained by enzymatic method before deproteinization and preincubation. *Conclusion:* Preincubation and deproteinization are the two simple approaches which can rectify the problem of negative interference of the bilirubin in estimating creatinine by Jaffe's method.

Keywords: Serum Creatinine, Pre incubation, High bilirubin, Deproteinization.

Introduction

Glucose and creatinine are the two most basic but essential test parameters. Accessibility to a lab in remotest rural areas is difficult. While glucose screening is easily available the same is not with the creatinine. Creatinine is a breakdown product of creatine phosphate. Its production is constant and depends on the muscle mass, age, sex, diet and exercise. The serum creatinine value is abnormal in cases of muscle and kidney diseases. Its estimation occurs as an important biochemical parameter in clinical laboratory tests [1]. Serum creatinine and serum bilirubin has been used in MELD score (Model for End-stage Liver Disease) [2]. Serum creatinine is also used in estimating Glomerular filtration rate (GFR). GFR is used in early diagnosis of kidney damage, chronic kidney disease and to monitor the renal function [3-4].

Bilirubin is the break down product of heme. 80% of the bilirubin is produced from the degradation of the hemoglobin present in erythrocytes which takes place in reticuloendothelial system. The another 20% is produced from the inefficient erythropoiesis in bone marrow and breakdown of other heme proteins. Unconjugated bilirubin is insoluble in water and it is transported to the liver in the blood by albumin. Inside the hepatocytes the conjugation of the bilirubin with glucuronic acid takes place and excreted in the bile [5].

It is also one of the important liver function tests. Both liver and kidney function test provide the knowledge about liver and kidney disease that occur in the same patient [6]. High performance liquid chromatography (HPLC) and gas chromatography with mass spectroscopy are the gold standard tests for creatinine estimation. But these methods are not used in most of the clinical laboratories since they are not readily available due to economic and technical constraints [7-8]. Hence this study was conducted to find out a possible solution to the problem of bilirubin interference. In this study we have tried to validate simple chemical method for serum creatinine estimation which is free from bilirubin interference. The performance of this method has been compared with the high performing enzymatic method.

The new method also helps in estimating the creatinine accurately in the presence of bilirubin interference in remotest villages where accessibility to the well established labs is difficult. The exact mechanism of bilirubin interference is not known. The interference may be due to the colour of the bilirubin affecting on the spectrum absorption with yellow colour of picrate used in creatinine estimation. Hence when creatinine has to be measured in samples containing high bilirubin colour produced by bilirubin should be removed or minimized [9].

In this study interference by the bilirubin is minimized by following two variations of Jaffe's kinetic method;

1) Preincubation of samples with NaOH for 5 min before the addition of Jaffe's reagent.
2) Deproteinization of the samples before creatinine estimation by Jaffe's method.

During preincubation NaOH oxidize bilirubin to biliverdin which will reduce the absorbance at 505nm, while we are trying to measure an increase in the absorbance at 505nm due to picrate creatinine interaction [6]. Deproteinization will remove the protein bound interfering substances such as bilirubin [9-10].

Material and Methods

This is an ICMR approved student project. Approval from the ethical committee was obtained from the concerned body before starting the study. This prospective comparative study was carried out in clinical biochemistry lab in tertiary care hospital for a duration of 6 months including 60 samples. Serum samples with bilirubin value >2 mg/dl were included in the study and hemolysed samples, lipemic samples, samples with high glucose concentration were excluded from the study.

Samples were divided into groups based on the bilirubin levels: Group I (1-5 mg/dl containing 20 samples), Group II (5-15mg/dl containing 20 samples), Group III (>15mg/dl containing 20 samples). Each group was divided into two subgroups of 10 samples each. Serum creatinine was estimated by enzymatic method and Jaffe's kinetic method. Total serum bilirubin was estimated by diazo method. Serum creatinine was measured once again in all the subgroups by Jaffe's kinetic method and enzymatic method after following variations.

1) Preincubation with NaOH for 5 min was done in all the subgroups containing 10 samples (10 samples in group I, 10 samples in group II, 10 samples in group III).
2) Deproteinization of the serum was done in all the subgroups containing 10 samples. (10 samples in group I, 10 samples in group II, 10 samples in group III).

Each serum sample was precipitated with 0.55mol/l of trichloro acetic acid at the ratio of 2:1 (sample volume: reagent volume). Serum sample is mixed with trichloroacetic acid and allowed to stand for 10 min. Centrifugation was done 1200g for 10 min. supernatant was separated and creatinine is estimated by both Jaffe's and enzymatic methods. The results were multiplied by 1.5 (dilution factor) [11].

The enzymatic assay of creatinine involves a series of enzymatic reactions. Creatininase converts creatinine to creatine. Creatinase converts creatine to sarcosine. Sarcoine oxidase converts sarcosine to formaldehyde, glycine and hydrogen peroxide. In the presence of peroxidase hydrogen peroxide is quantified by the formation of coloured dye [12]. Serum creatinine thus obtained after the variations of Jaffe's kinetic method was

compared with serum creatinine obtained by enzymatic method. Pooling of the all serum samples having high bilirubin levels was done for one month. Samples were stored in deep freezer at -200 C until the analysis.

Statistical analysis:

1) Results were tabulated. Mean, standard deviation, range of creatinine was calculated in all the groups.
2) Comparison of serum creatinine between groups was done by t test.

Results

Table no. 1 shows comparison of creatinine values in group I with bilirubin <5mg/dl. This table shows that there is significant difference in the creatinine value obtained by Jaffe's kinetic method before and after deproteinization and preincubation.

There is also significant difference in the creatinine value obtained by enzymatic method before and after deproteinization and preincubation.

Table- 1: Comparison of creatinine values in group I with bilirubin <5mg/dl

Subgroups	Mean ± SD	Range	P value
Creatinine by Jaffes method before deproteinization	0.88 ± 0.27	0.38-1.22	0.001
Creatinine by Jaffes method after deproteinization	1.06 ± 0.30	0.6-1.54	
Creatinine by enzymatic method before deproteinization	1.036 ± 0.33	0.49-1.55	0.005
Creatinine by enzymatic method after deproteinization	1.21 ± 0.37	0.7-1.85	
Creatinine by Jaffes method before Preincubation	0.847 ± 0.28	0.39-1.22	P<0.0001
Creatinine by Jaffes method after preincubation	1.008 ± 0.32	0.52-1.5	
Creatinine by enzymatic method before preincubation	1.086 ± 0.26	0.79-1.55	P<0.0001
Creatinine by enzymatic method after preincubation	0.63 ± 0.29	0.32-1.18	

Table No. 2 shows comparison of creatinine values in group II with bilirubin 5-15 mg/dl. This table shows that there is significant difference in the creatinine value obtained by Jaffe's kinetic method before and after deproteinization and preincubation. There is also significant difference in the creatinine value obtained by enzymatic method before and after deproteinization. But there is no significant difference in the creatinine value obtained by enzymatic method before and after preincubation.

Table-2: Comparison of creatinine values in group II with bilirubin 5-15 mg/dl

Subgroups	Mean ± SD	Range	P value
Creatinine by Jaffes method before deproteinization	0.59 ±0.17	0.37-0.9	0.002
Creatinine by Jaffes method after deproteinization	0.713±0.16	0.51-0.96	
Creatinine by enzymatic method before deproteinization	0.75±0.14	0.58-0.98	P<0.0001
Creatinine by enzymatic method after deproteinization	1.03±0.18	0.70-1.36	
Creatinine by Jaffes method before preincubation	0.641±0.24	0.12-0.98	0.007
Creatinine by Jaffes method after preincubation	0.98±0.48	0.23-1.94	
Creatinine by enzymatic method before preincubation	0.635±0.22	0.2-0.95	0.13
Creatinine by enzymatic method after preincubation	0.319±0.58	0.01-1.62	

Table No. 3 shows comparison of creatinine values in group III with bilirubin >15 mg/dl. This table shows that there is significant difference in the creatinine value obtained by Jaffe's kinetic method before and after deproteinization and preincubation. There is also significant difference in the creatinine value obtained by enzymatic method before and after deproteinization and preincubation.

Table-3: Comparison of creatinine values in group III with bilirubin >15 mg/dl.			
Subgroups	**Mean ± SD**	**Range**	**P value**
Creatinine by Jaffes method before deproteinization	0.248 ±0.13	0.04-0.53	P<0.0001
Creatinine by Jaffes method after deproteinization	0.715±0.12	0.52-0.9	
Creatinine by enzymatic method before deproteinization	0.696 ±0.10	0.5-0.85	0.0001
Creatinine by enzymatic method after deproteinization	1.02 ± 0.16	0.77-1.32	
Creatinine by Jaffes method before Preincubation	0.335 ±0.23	0.04-0.74	P<0.0001
Creatinine by Jaffes method after preincubation	0.64 ±0.23	0.32-0.96	
Creatinine by enzymatic method before preincubation	0.728 ±0.25	0.45-1.2	P<0.0001
Creatinine by enzymatic method after preincubation	0.053 ± 0.04	0.01-0.13	

Table No. 4 shows comparison of creatinine values in group I, group II, group III. This table shows that there is no significant difference in the creatinine value obtained by Jaffe's method after deproteinization and creatinine obtained by enzymatic method before deproteinization in all the three groups. It also shows that there is no significant difference in the creatinine value obtained by Jaffe's method after preincubation and creatinine obtained by enzymatic method before preincubation in all the three groups.

Table-4: Shows comparison of creatinine values in group I, group II, group III					
Groups	**Total bilirubin level**	**Subgroups**	**Mean ± SD**	**Range**	**P value**
I	<5mg/dl	Creatinine by jaffes method after deproteinization	1.06 ± 0.30	0.6-1.54	0.62
		Creatinine by enzymatic method before deproteinization	1.036±0.33	0.49-1.55	
		Creatinine by jaffes method after preincubation	1.008±0.32	0.52-1.5	0.34319
		Creatinine by enzymatic method before preincubation	1.086±0.26	0.79-1.55	
II	5-15 mg/dl	Creatinine by jaffes method after deproteinization	0.713±0.12	0.51-0.96	0.4703
		Creatinine by enzymatic method before deproteinization	0.746±0.14	0.58-0.98	
		Creatinine by jaffes method after preincubation	0.98±0.48	0.23-1.94	0.06
		Creatinine by enzymatic method before preincubation	0.635±0.22	0.2-0.95	
III	>15 mg/dl	Creatinine by jaffes method after deproteinization	0.715±0.12	0.52-0.9	0.480692
		Creatinine by enzymatic method before deproteinization	0.696±0.09	0.5-0.85	
		Creatinine by jaffes method after preincubation	0.643±0.23	0.32-0.96	0.3108
		Creatinine by enzymatic method before preincubation	0.728±0.25	0.45-1.2	

Table No. 5 shows comparison of creatinine values in group I, group II, group III. This table shows that there is significant difference in the creatinine by Jaffe's method before deproteinization and preincubation and creatinine value obtained by enzymatic method before deproteinization and preincubation in all the groups.

Table-5: Shows comparison of creatinine values in group I, group II, group III					
Groups	**Total bilirubin level**	**Subgroups**	**Mean ± SD**	**Range**	**P value**
I	<5mg/dl	Creatinine by jaffes method before deproteinization and preincubation	0.863 ± 0.27	0.38-1.22	0.0001
		Creatinine by enzymatic method before deproteinization and preincubation	1.061±0.29	0.49-1.55	
II	5-15 mg/dl	Creatinine by jaffes method before preincubation and deproteinization	0.62 ±0.203	0.12-0.98	0.0265
		Creatinine by enzymatic method before deproteinization and preincubation	0.690±0.1868	0.2-0.98	
III	>15 mg/dl	Creatinine by jaffes method before preincubation and deproteinization	0.292±0.188	0.04-0.74	<0.0001
		Creatinine by enzymatic method before deproteinization and preincubation	0.712 ±0.18	0.45-1.2	

Discussion

The Jaffe's method is used in clinical labs for estimation of serum creatinine. But it is well known that bilirubin will negatively interfere with estimation of serum creatinine by Jaffe's method [11]. Kinetic method does not include precipitation of proteins which also removes the bilirubin [13]. Both conjugated and unconjugated bilirubin cause the apparent decrease in the serum creatinine [14]. There are several techniques to overcome bilirubin interference on serum creatinine by the Jaffe's kinetic method. In this study interference by the bilirubin is minimized by following two variations of Jaffe's kinetic method

1) Preincubation of samples with NaOH for 5 min before the addition of Jaffe's reagent.
2) Deproteinization of the samples before creatinine estimation by Jaffe's method.

At present creatinine estimation by enzymatic method has been accepted as one of the most accurate routine methods. Enzymatic method is suitable for the measurement of serum creatinine particularly in diabetic ketotic patients, neonates and patients receiving cephalosporins [15]. There are several advantages of enzymatic method of measuring serum creatinine over Jaffe's kinetic method. They are improved specificity, smaller sample volume and hence a rapid sample throughput. Glucose, acetoacetate and Cefoxitin do not interfere with enzymatic method. But bilirubin will negatively interfere which depends on the both creatinine and bilirubin concentrations [16].

Although enzymatic method is effective in estimating creatinine in the presence of most of the interfering substances it has greater cost and shorter halflife compared with kinetic Jaffe's method [17]. In cases of neonates and adults having high bilirubin value creatinine is underestimated by Jaffe's kinetic method [15]. Enzymatic method of creatinine estimation gives the reliable results when the samples take time to reach the lab and blood centrifugation is delayed for 24 hours or more. It has been shown in recently published study that there was false increase in the serum creatinine by alkaline picrate method when specimens take time to reach the lab and blood centrifugation is delayed for 24 hours or more. This may be due to the interference by some metabolites built up in vitro like pyruvate and ketones [18].

Our study shows that there was significant improvement in values of creatinine estimated by Jaffe's method after deproteinization as compared to values before deproteinization. P value in group I was 0.001, in group II was 0.002, in group III was <0.0001. P value for group I and Group II was significant but for group III it was found to be highly significant. From the findings of our study it is shown that there is a significant improvement in the creatinine estimated by Jaffe's method after deproteinization as compared to the values before deproteinization. These findings are in accordance with the studies by Nigam PK, Lolekha PH et al, Parmar V et al and Lee SY et al [11, 13, 19-20].

The best approach to correct the bilirubin interference on serum creatinine estimation is either through the deproteinization with acid [21-22] or by separating the albumin bilirubin complex through the membrane layer [23]. Our study shows that there was significant improvement in creatinine obtained after preincubation as compared to values before preincubation. P value for group I and group III was<0.0001 and for group II was 0.007. These findings are in agreement with previous studies done by Chauhan S et al, Nigam PK. [6, 11].

In our study it was found that there was better response in creatinine estimation by Jaffe's method after preincubation as compared to creatinine estimated after deproteinization. This is in accordance with the study done by Nigam PK [11]. It is found that creatinine value obtained by Jaffe's method after deproteinization and preincubation are not significantly different from creatinine obtained by enzymatic method before deproteinization and preincubation. In a similar study by Lee SY et al it was found no significant difference between creatinine estimated by Jaffe's method after deproteinization and enzymatic method [20]. This study shows that there is a significant difference in the creatinine obtained by Jaffe's method when compared to enzymatic method. These findings are in accordance with studies done by Malukar NR [15] Sridhar K et al [16]. But studies done by Gencheva II [24] and Marakala V [17] are not in agreement with our study.

There was significant difference between the creatinine values obtained by enzymatic method before and after deproteinization. These findings suggest that even enzymatic method will be negatively interfered by bilirubin since there is improvement in the creatinine obtained after removal of the bilirubin through deproteinization. The interference of bilirubin in creatinine estimation by peroxidase coupled enzymatic method of determination of creatinine has been explained by both spectral and chemical effects. Spectral interference is due to the overlapping of spectra of bilirubin and chromophore. Chemical interference is due to the destruction of the part of the reactive intermediate formed in the peroxidase reaction by bilirubin which decreases the amount of the chromophore formed [25].

The findings from the study suggest that there is decrease in the creatinine values obtained by enzymatic method after preincubation as compared to before preincubation. The reasons for decrease in the creatinine values obtained after preincubation by enzymatic method may be alteration in PH due to the preincubation of the sample with NaOH which will affect the activity of the enzymes, denaturation of the enzymes by NaOH decreasing their activity.

Conclusion

Thus it is concluded from our study that preincubation and deproteinization are the two simple approaches which can rectify the problem of negative interference of the bilirubin in estimating creatinine by Jaffe's method. The creatinine obtained after these approaches by Jaffe's method are similar to creatinine obtained by enzynmatic method before deproteinization and preincubation. Thus from this study we have validated simple chemical method that is Jaffes method for serum creatinine estimation which is free from bilirubin interference. The performance of this method has been compared with the high performing enzymatic method.

The new method also helps in estimating the creatinine accurately in the presence of bilirubin interference in remotest villages where accessibility to the well established labs is difficult. A larger sample size may be required to validate the findings of our study. Similar studies can be done to see the effect of the glucose, hemolysis, lipemia and other interfering substances on the Jaffe's method and enzymatic method. Studies are to be undertaken to compare the creatinine values obtained by Jaffe's method after the deproteinization or preincubation with creatinine estimated by high performance liquid chromatography and gas chromatography with mass spectroscopy

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*All correspondences to: Dr. Thivyahprabha AG, Associate Professor, Department of Biochemistry, St.Peter's Medical College Hospital & Research Institute, Opposite to SIPCOT 2, Hosur-635109, Tamilnadu, India. E-mail: drthivyahprabha@gmail.com