Indian Journal of Experimental Biology
Vol. 40, March 2002, pp. 352-354

Measurement of creatinine by Jaffe's reaction — Determination of concentration of sodium hydroxide required for maximum color development in standard, urine and protein free filtrate of serum

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Received 13 November 2000; revised 30 October 2001

Creatinine in serum or urine is determined by Jaffe's reaction where creatinine produces quantitatively an orange color with picric acid in alkaline medium. After allowing an incubation time of 15 min at room temperature for color development the color is measured at 520nm. Without taking into consideration the acidic nature of standard, protein free filtrate (PFF) of serum and urine, 1% picric acid and 0.75*N* NaOH are used in this reaction for color development in standard, PFF of serum and urine. An investigation was thought to be necessary to determine the optimum alkali concentration required in standard, PFF of serum and urine. The results show that 0.25, 0.75 and 1*N* NaOH give maximum color in urine, standard and PFF of serum respectively. A standard solution of creatinine is prepared in 0.1*N* HCl and the PFF of serum is obtained by addition of fresh tungstic acid . Alkali is consumed to neutralise the acids in both these cases. For urine creatinine measurement, a direct diluted urine sample is used .The difference in the requirement of NaOH is conceivable. The routine use of 0.75*N* NaOH irrespective of the nature of specimen as is done in all biochemical laboratories, for creatinine measurement needs modification in the light of this investigation.

Measurement of creatinine in serum is a renal function test[1]. Its normal range in serum is 0.5-1.5mg/dl. Due to the low value, an accurate measurement is warranted in the assessment of renal dysfunction. The determination of creatinine in serum or urine is carried out by Jaffe's reaction where creatinine reacts with picric acid in an alkaline medium[2]. The alkalinity is provided by sodium hydroxide.

As a routine, clinical biochemistry laboratories use 0.75*N* NaOH and 1% picric acid at a volume of 1ml each to determine creatinine in protein free filtrate of serum or diluted urine[3]. The standard is similarly treated. Creatinine standard is prepared in 0.1*N* HCl as it is insoluble in water. The yellowish orange color developed within 15 min at room temperature is measured at 520nm in a photometer[4]. What is not taken into account is the acidic nature of the protein free filtrate (PFF) of serum or acidic nature of the standard may consume part of the NaOH added thereby diminishing the alkali essential for color development. The present investigation shows the adequacy of NaOH ideal for color development in creatinine standard, protein free filtrate and fresh diluted urine.

Following chemicals were used:

Fresh unhemolysed serum.

Freshly voided urine

Creatinine standard (stock) - Analytical grade creatinine (100 mg) was dissolved in 0.1*N* HCl in a standard flask and made upto 100 ml with the acid and mixed.

Creatinine working standard - Stock solution (1ml) of creatinine was diluted to 100 ml with water (0.01mg/ml).

Picric acid (1%) — Recrystallised picric acid (10g) was dissolved in water and made up to 1 litre with water.

Sodium hydroxide solution of 0.25*N* (1%): 0.5*N* (2%): 0.75*N* (3%) and 1.*N* (4%) were prepared from analytical grade sodium hydroxide in water.

Experiment no. 1

Determination of optimum concentration of NaOH required for maximum color development in standard — Two test tubes were labeled blank (B) and standard (S). Reagents were taken in B and S as per the following schedule:

Reagents (ml)	B	S
Creatinine workng standard	—	2.5
Water	4	1.5
0.75*N* NaOH	1	1
1% Picric acid	1	1

The tubes were incubated at room temperature after mixing for 15 min. The photometric readings were taken at 520 nm with water blank. The absorbance of standard and blank were noted.

The experiment was repeated replacing 0.75*N* NaOH by 0.25, 0.5 and 1*N* NaOH. Absorbance in each case was noted. The analysis was carried out with five samples.

Experiment no. 2

Determination of NaOH concentration required for maximum color development for measurement of creatinine in urine — *Freshly* voided urine was diluted 1 in 100 in a standard flask with distilled water. The two test tubes were labeled blank (B) and test (T) and proceeded as follows:

Reagents (ml)	B	T
Diluted urine	—	4
Water	4	—
0.75*N* NaOH	1	1
1% Picric acid	1	1

The contents were mixed and incubated at room temperature for 15 min. The absorbance of B and T were taken at 520 nm with water blank.

The experiment was repeated with NaOH concentrations of 0.25, 0.5, and 1*N*. The absorbance value in each case was noted. The analysis was carried out with ten different urine samples.

Experiment no. 3

Determination of NaOH concentration required for maximum color development for measurement of creatinine in serum.

Preparation of protein free filtrate—To 0.5 ml serum taken in a centrifuge tube, 1.5 ml water, 1 ml of 10% sodium tungstate and 1 ml of 2/3*N* sulphuric acid were added, mixed and centrifuged. The protein free supernatant was transferred into another dry tube.

Color development—Two test tubes were labeled blank (B) and test (T) and proceeded as follows:

Reagents (ml)	B	T
Protein free filtrate	—	1
Water	4	3
0.75*N* NaOH	1	1
1% Picric acid	1	1

The contents were mixed and incubated for 15min at room temperature. The absorbance of B and T were taken at 520 nm with water blank. The experiment was repeated with NaOH concentrations of 0.25, 0.5 and 1*N*.

The absorbance — blank value in each case was noted.

The analysis was carried with 10 different serum samples. *P* value is calculated by one way ANOVA test and Turkey's multiple comparison test.

The results are given in Table 1.

NaOH concentration required was 0.25*N*(1%) for urine, 0.75*N* (3%) for standard and 1*N* (4%) for serum in the procedure followed in this investigation.

Creatinine determination in biological fluids was carried out by Jaffe's reaction. The exact mechanism of color development is not clear. It is believed to be due to the formation of creatinine picrate in the alkaline medium .As a routine, an equal volume of 3% NaOH (0.75*N*) and 1%picric acid are used in the color development without taking into account the *p*H or acidity of the reacting fluid. In urinary creatinine measurement, it was a direct sample (diluted) but in serum it was an acidic protein free filtrate. The creatinine standard also was prepared in 0.1*N* HCl. The *p*H of the diluted urine, serum (PFF) and working standard was 7.20, 2.50 and 6.12 respectively (Table 2). Obviously the acidity will neutralise the NaOH added to some extent which may influence the reaction .The results clearly show that the concentration of NaOH is important in the reaction to develop maximum color.

During the study, the effect of incubation time and temperature were also investigated and it was observed that 15 min time was required at room tem-

Table 1—Effect of different concentrations of NaOH on absorbance (maximum color development)

[Values are mean ± SE]

Concentration of NaOH (*N*)	Absorbance		
	Standard	Serum	Urine
0.25(1%)	0.082 ± 0.001	0.022 ± 0.0019	0.143 ± 0.019^{NS}
0.50 (2%)	0.118 ± 0.001	0.032 ± 0.003	0.140 ± 0.018
0.75(3%)	0.190 ± 0.002*	0.037 ± 0.003	0.125 ± 0.017
1(4%)	0.112 ± 0.002	0.044 ± 0.002*	0.117 ± 0.002

P value: * < 0.001

Tabel 2—Effect of concentration of NaOH on *p*H of urine, serum (PFF) and standard samples
[Figures in parenthesis are % increase]

Sample	*p*H before NaOH addition	*p*H after NaOH addition			
		1 %	2 %	3 %	4 %
Urine	7.20	12.47 (73.19)	12.55 (74.30)	12.62 (75.27)	12.70 (76.38)
Serum(PFF)	2.50	12.25 (390.0)	12.40 (396.0)	12.45 (398.0)	12.53 (401.2)
Standard	6.12	12.30 (100.9)	12.42 (102.9)	12.49 (104.1)	12.61 (106.0)

perature (30°C). At 37°C in a water incubator also the time factor remained the same as 15 min. The desirable *p*H of reacting fluids after adding all the reagents was found to be around 12.5 in standard, urine and serum, after the suggested modifications of using 0.75*N*, 0.25*N* and 1*N* respectively (Table 2).

Since creatinine measurement in serum is very important in a hospital laboratory for diagnosis, prognosis and in emergency as a renal function parameter, any methodological error in measurement cannot be ignored.

Reference

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