Indian Journal of Science and Technology, *Vol 9(4), DOI: 10.17485/ijst/2016/v9i4/80499, January 2016*
ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

New Method for Assessment of Serum Catalase Activity

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Abstract

Background: The following study presents a colorimetric method for the assessment of serum catalase activity which yields precise, accurate, reproducible results and is simplified so that clinical pathology laboratories may achieve this determination without the need for special techniques. **Methods**: In this method, dichromate in acetic acid is reduced to chromic acetate when heated in the presence of undecomposed hydrogen peroxide (H_2O_2), with the formation of perchromic acid as an unstable intermediate. Hydrogen peroxide concentration is directly proportional to the concentration of chromic acetate that produced from the reaction. The chromic acetate produced is measured calorimetrically at 570 nm. **Findings:** The imprecision of the method was calculated by measuring the coefficient of variation, which equals to 3.4% within run and 5.9% between run. The catalase assay performed using the kinetic method yielded a good correlation (r = 0.9771). **Applications:** The present method characterizes by adding a correction factor to eliminate the interference that arises from the presence of sugars, amino acids, proteins and vitamins in serum.

Keywords: Catalase Activity, Clinical Pathology, New Method Serum, Spectrophotometry

1. Introduction

The rapid development of clinical pathology form of payments to all branches of enzymology, including analytical assessment[1,2]. Rapid methods for the estimation of catalase activities in the active field of applied biochemistry are increasing due to the broad importance of catalase in clinical pathology. Serum catalase (EC 1.11.1.6) activity is increased in acute pancreatitis for a considerably longer time than the serum amylase activity and is also recorded to be raised in chronic pancreatitis[3]. Although this has been recognized for several decades, serum catalase activity has achieved little attractiveness because of the lack of simple and reliable methods for its assessment[4].

Catalase can be assessed by determining the rate of decomposition of H_2O_2 (at 240 nm)[5]. There are practical difficulties with this method, which belonging to using very high and un-physiological levels of substrate (5-50 mM) for attaining acceptable initial absorbance (the absorbance of H_2O_2 at 240 nm is only 39.4 M^{-1} cm^{-1})[6]. These high levels of H_2O_2 lead to rapidly but variable auto-inactivation of catalase by modification of the active enzyme - H_2O_2 complex I to the inactive complex II. Moreover, many cellular components such as proteins absorb strongly at 240 nm so that low activities of catalase often have to be measured by the continuous method against an elevated background absorbance[5]. Other documented methods for determining catalase activity involve titrimetric determination of hydrogen peroxide, determination of oxygen production by oxygen electrode, immune-precipitation and gasometric determination of hydrogen peroxide[8,9]. These methods are tedious and not suitable for clinical use.

Modern assays of catalase include three methods. The first depends on the enzymatic consumption of hydrogen peroxide using INH-PC [Iso-Nicotinicacid Hydrazide-Pyrocatechol] system[10]. The using of H_2O_2, INH-PC reagent system produced a chromogenic complex with

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maximum absorbance at 490 nm. The second method utilized amperometric flow injection unit for measuring of undecomposed hydrogen peroxide[11]. These types of assays need special laboratory equipment to complete. Goth[12] was described a simple colorimetric assay for catalase, in which the measuring of un-reacted hydrogen peroxide is measured spectrophotometrically by a complex reaction with ammonium molybdate. In the third method, Hadwan et al.[13] introduced some modifications to the Goth method to increase confidence, sensitivity and precision of Goth method. They utilized a certified standard enzyme with known unit activity as an alternative of necessitation to calibrate precise H_2O_2 concentration to 30 mM in a complex and tedious process. Calibration of precise H_2O_2 concentration is not required in the modified method because catalase activity in the sample is determined by comparing with standard catalase enzyme. The disadvantage of this method includes the difficulty of obtaining a certified catalase enzyme with known activity.

Sinha[14] was described a simple colorimetric assay for catalase, in which the decomposition of peroxide is estimated spectrophotometrically by a complex reaction with dichromate/acetic acid reagent without using the optimized conditions and taking into account the effects of pathologic, i.e. icteric, lipemic, hemolytic and diabetic sera. We now report a modified method which combines the spectrophotometric assay of hydrogen peroxide with an optimized serum catalase determination. The present method acts to eliminate the interference arising from the presence of sugars, fats, amino acids and proteins in the sample containing catalase enzyme.

2. Materials and Methods

2.1 Principle

Catalase activity was determined according to the method described previously by Sinha[14]. In this method, dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), with the formation of perchromic acid as an unstable intermediate. Hydrogen peroxide concentration is directly proportional to the concentration of chromic acetate that produced from the reaction. The chromic acetate produced is measured calorimetrically at 570 nm.

$$Cr_2O_7^{-2} + 7H_2O_2 \rightarrow 2CrO_8^{-3} + 5H_2O + 4H^+$$

Blue perchromic acid

$$2CrO_8^{-3} + 6CH_3COO^- + 6H^+ \rightarrow 2Cr(CH_3COO)_3 + 14H_2O$$

Chromic acetate (λ_{max} = 570 nm)

2.2 Instrument

A spectrophotometers (PG Instruments T80) and (Shimadzu 1800 spectrophotometer) were used in the study.

2.3 Chemicals

All reagents and chemicals were of analytical grade and obtained from standard commercial suppliers.

2.4 Reagents

- Dichromate/acetic acid: This reagent is prepared by mixing 50 ml of a 5% aqueous solution of potassium dichromate with 150 ml of glacial acetic acid.
- Sodium, potassium phosphate buffer (50 mM, pH 7.4): This buffer is prepared by dissolving 1.1 g of Na_2HPO_4 and 0.27 g of KH_2PO_4 in 100 ml distilled water containing 0.1% w/v bovine serum albumin.
- H_2O_2 (65 mM) in 50 mmol/L sodium, potassium phosphate buffer: This solution is freshly diluted and standardized daily using a molar extinction coefficient of 39.4 M^{-1} cm^{-1} at 240 nm.

2.5 Procedure

Enzyme activity according to procedure descriping in Table 1.

Table 1. Shows the procedure that used for measurement of catalase activity

Reagents	Test	Control-test*	Standard	Blank
Serum	100 µl	100 µl	------	-----
Distilled water	------	1000 µl	100 µl	1100 µl
Hydrogen peroxide	1000 µl	------	1000 µl	-----
Mix with vortex and incubate at 37 °C for 3 min, after that, add:				
Dichromate/ acetic acid	2000 µl	2000 µl	2000 µl	2000 µl

After that, the tubes were kept at 100 °C for 10 min. After cooling with tap water, centrifuged to remove precipitated protein (2500 g for 5 min), the changes in absorbance were recorded at 570 nm against the reagent blank.

2.6 Calculation

The following equation is used to determine catalase activity:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * \left[\log \frac{S^o}{S-M}\right] * \frac{Vt}{Vs}$$

t: time.
S^o: Absorbance of standard tube.
S: Absorbance of test tube.
M: Absorbance of control test (correction factor).
Vt: Total volume of reagents in test tube.
Vs: Volume of serum.

* The present method characterizes by adding a correction factor (Control-test) to eliminate the interference that arises from the presence of sugars, amino acids, proteins and vitamins in serum. The absorbance of test tube in procedure is belonging to two types of compounds, un-reacted hydrogen peroxide and interferences found in serum. The absorbance of (Control-test) tube in procedure is belonging to interferences compounds found in serum only. By subtract the absorbance of (Control-test) tube from the absorbance of test tube; we remove the interference of oxidizable compounds such as free sugars and basic amino acid. That is mean, the remaining absorbance belonging to un-reacted hydrogen peroxide only.

3. Results and Discussion

An absorbance spectrum of the colorimetric products was measured from 400 nm to 700 nm and revealed a peak at 570 nm (Figure 1), suggesting the optimum colorimetric wavelength for the green reaction product was 570 nm.

The dichromate/acetic acid reagent can be considered as a "stop bath" for enzymatic reaction that catalyzed by catalase enzyme. As soon as the enzyme reaction solution mixes the acetic acid, its molecules are denatured; any hydrogen peroxide which hasn't been decomposing by the catalase will react with the dichromate to produce a blue precipitate of perchromic acid. This unstable precipitate is then dissolved by heating to generate the green solution of chromic acetate, which has a maximum absorbance at 570 nm. As Sinha[14] declares, a problem with the dichromate/acetic acid method is that sugars and basic amino acids will also react with dichromate; however, the author indicates that these molecules shouldn't have significant effects at the levels of the enzyme that scientific

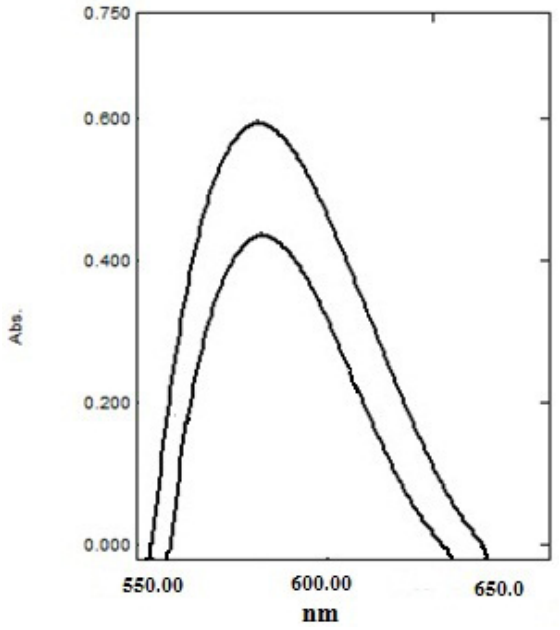

Figure 1. An absorbance spectrum of the colorimetric product that measured from 400 nm to 700 nm.

research will be dealing with. In fact, Sinha method can avoid the subject of interference altogether if researchers use as pure a catalase preparation as possible. In practice, Sinha method cannot be used to measure the actual catalase enzyme activity in serum because there are many interference molecules in serum that lead to get results is quite unreliable. Interference molecules include uric acid, ascorbic acid, glucose, amino acids, protein, bilirubin, etc. This has led us to find a simple method suitable for serum as well as other biological samples.

The present method characterizes by adding a correction factor (Control-test) to eliminate the interference that arises from the presence of sugars, amino acids, proteins and vitamins in serum. To investigate the effect of interferences that might perturb the catalase assay, ten volumetric flasks, to each one was added one ml of catalase known activity (500 kU/l) [obtained from Himedia (Product Code: TC037). Its activity was calibrated according to Aebi's method[3] and nine ml of 55.55 µmol/l of one interference only that dissolved in phosphate buffer (50 mM, pH 7.4). The final activity equals to 50 kU/l of catalase with 50 µmol/l of interference. Catalase enzyme activity was measured by Sinha method and the results compared with that obtained by present method. Table 2 shows the effects of various interferences on the catalase assay. Catalase enzyme activity was not significantly

Table 2. Shows the effects of various interferences on the catalase assay

Substance	Concentration of substance	Observed Catalase activity			
		Sinha method	Recovery %	Present method	Recovery %
-----	0	49.27	-----	49.33	-----
Glucose	50	47	94	49.6	99.2
Fructose	50	47	94	49.75	99.5
Cysteine	50	44.55	89.1	49.65	99.3
Histdine	50	28.85	57.7	49.6	99.2
Lysine	50	26.5	53	49.55	99.1
Ascorbic acid	50	25.35	50.7	49.55	99.1
Uric acid	50	32.1	64.2	49.1	98.2
Cholesterol	50	36.3	72.6	49.35	98.7
Triglyceride	50	49.55	99.1	49.65	99.3

Table 3. Precision of the assay procedure

	No.	Mean (±SD): k.U/liter	CV %
Within-run	20	110.7 ± 3.8	3.4 %
Between-run	20	106.7 ± 6.4	5.9 %

affected by a considerable amount of each interference compound when measured by present method. However, interferences affected catalase enzyme activity when used Sinha method.

Results obtained from serum by this method were compared with that obtained by the method of Aebi[5]. Identical sample, buffer and substrate concentrations were used in both methods. The results of the present assay proved a good precision (Table 3) and a good correlation with the Aebi's Method (Table 4).

Accuracy of the entire assay protocol was measured by recovery of hydrogen peroxide added to the reaction solution after the end of enzymatic reaction detailed in the Table 5.

The study clarifies a simple discontinuous assay for serum catalase activity which can be achieved with few steps and which allows catalase to be measured in the presence of high levels of other molecules as well as at low, 'near-physiological' levels of H_2O_2. The dichromate/acetic acid reagent is a sensitive assay for H_2O_2 which thus permits measurement of catalase at low levels of substrate ensuring that auto-inactivation of catalase is minimized during the process of measurement.

Table 4. Statistical analysis of the values obtained for catalase by Aebi's method and present method

No. of Samples	20
Mean of Aebi's Method	118
Mean of the present method	112
Mean of both methods	115
Regression coefficient B	0.9723
Regression coefficient A	0.0277
Correlation coefficient	0.9771

Table 5. Analytical recovery of hydrogen peroxide added to the reaction solution after the end of enzymatic reactions

Present in assay	Equivalents of hydrogen peroxide	Calculated activity kU/liter	Observed activity[a] kU/l	Recovery %
Enzymatic sample	-----	-----	50	-----
Enzymatic sample + hydrogen peroxide	10	60	61	101 %
Enzymatic sample + hydrogen peroxide	25	75	73	98 %
Enzymatic sample + hydrogen peroxide	50	100	97	98 %
Enzymatic sample + hydrogen peroxide	100	150	144	96 %
Enzymatic sample + hydrogen peroxide	200	250	241	96 %

[a] mean of triplicate determinations

In conclusion, we have developed a simple and cost-effective assay. We demonstrated its feasibility in determining the serum catalase activity. The current assay presents a number of advantages more than existing methodologies. These advantages include more precision and accuracy, instrumentals and apparatus are not complicated and available in most laboratories and the assay is free from interference.

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