ORIGINAL ARTICLE

Development of Novel Bienzymatic Assay for Quantification of Glucose in Human Serum

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Abstract

Aim: The main objective of this investigation was to design and to develop a simple, sensitive, and convenient assay for quantification of glucose in human serum sample. **Materials and Methods:** A novel bienzymatic assay for the quantification of glucose by spectrophotometry has been developed. The method is based on the coupling of 4-aminoantipyrine and iminodibenzyl in the presence of bienzyme peroxidase/glucose oxidase (GO_x) to form a blue colored product which is monitored for absorbance at 620 nm at 23°C in 100 mmol/L KH_2PO_4/NaOH buffer of pH 6.3. **Results and Discussion:** Under optimized assay conditions, glucose could be quantified in a linear range from 1.15 to 562 µmol/L and 2.31 to 370 µmol/L by kinetic and fixed time method. The limit of detection and limit of quantification were measured. Michaelis–Menten constant for glucose was found to K_m = 357 µmol/L and maximum rate of the reaction (V_{max}) was 0.117 × 10^{-6} mol/L/min. The proposed method was applied for the quantification of glucose in human serum samples, which has a good regression coefficient of 0.9992 with the enzymatic kit method. The inter-day precision was 0.53-2.9% (n = 10) and intra-day precision was 1.5-3.03% (n = 10). The accuracy range for glucose was fond to be 90-104%. The glucose recovery ranged from 91.92% to 108.5%. **Conclusion:** The present assay was rapid, sensitive, and convenient for quantification of glucose in human serum sample with good recovery, high accuracy, and minimum interference.

Keywords: Glucose assay, human serum sample, hydrogen peroxide assay, Michaelis–Menten constant

INTRODUCTION

Diabetes is a serious lifestyle-related disease that can result in a myriad of complications including heart disease, kidney failure, and blindness.[1] Hence, exact, rapid and quantitative determination of glucose level with high sensitivity and reproducibility is very essential in the diagnosis and management of diabetes. Numerous methods have been developed for precisely monitoring the glucose level with high specificity, high reliability, fast response, good selectivity, and low cost.[2,3] These include chemiluminescence,[4,5] fluorescence,[6] electrochemical,[7-9] sequential injection spectrophotometry,[10] chromatoghaphy,[11] and spectrophotometry.[12,13]

Each method has its own merits and demerits. A sensitive chemiluminescence method was reported for glucose determination in the range of 0.01-1 mmol/L,[4] but some metal ions catalyzed the luminol chemiluminescence reaction besides the instruments used are expensive, less versatile, selectivity and sensitivity of the luminesence is poor. An intrinsic fluorescence method has been reported for the determination of 0.5-20 mmol/L glucose but low sensitivity is the defect.[6] The electrochemical method has good sensitivity for quantification of glucose range from 8 × 10^{-4} to 2 × 10^{-2} mmol/L[9] but it has several steps for immobilization of enzyme on electrode, which may reduce the enzyme activity and also during incorporation of enzyme on electrode a large amount of expensive biocatalyst gets wasted. A sequential injection spectrophotometer could be used for the quantification of glucose up to 0.69 mmol/L.[10]

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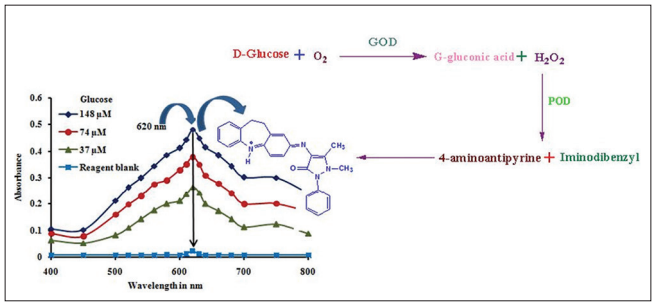
The method is simple and convenient but has less sensitivity. Many cosubstrates have been used for the quantification of glucose,[12-16] but these reagents have some limitations such as carcinogenicity, mutagenicity, less solubility, broad linearity range, and poor sensitivity. In addition, most of the methods have not taken into consideration the commonly interfering species present in blood composition like maltose, lactose, fructose, galactose, and sucrose and the incubation time for the reaction mixture was exceeded 10 min.

In this study, a new method with simplified procedure to overcome some of the above limitations has been proposed. It is based on the principle that enzymatic oxidation of glucose produces gluconic acid and hydrogen peroxide in the presence of glucose oxidase (GO_x).[4] This hydrogen peroxide reacts with 4-aminoantipyrine (4-AAP) and iminodibenzyl (IDB) under the catalytic influence of peroxidase (POD) to yield an intense blue colored product that shows strong absorbance at 620 nm [Scheme 1].

MATERIALS AND METHODS

Chemicals

POD (EC.1.11.1.7, 100 units/mg) was purchased from Himedia Laboratories (Mumbai, India). The stock solution of POD with a concentration of 0.4545×10^{-5} mol/L was prepared in 100 mmol/L potassium dihydrogen orthophosphate/sodium hydroxide (KH_2PO_4/NaOH) (LR, Rankem, New Delhi, India) buffer solution of pH 6.0. The enzyme solution was stored in refrigerator at 4°C. Working solutions were prepared from the stock solution by dilution with water. The standard stock solution of H_2O_2 (1%, v/v) was prepared daily by diluting the analytical reagent grade reagent (30%, v/v, E. Merck, Mumbai, India) and standardized by potassium permanganate (99%, LR, Thomas Baker Chemicals, Mumbai, India) method. Working solutions of H_2O_2 were prepared by successive dilution of the standard solution with distilled water. Highly purified GO_x (EC 1.1.3.4, from *Aspergillus niger*, 200 U/mg, Sigma) stock solution with 150 U/mg activity was prepared using distilled water. Glucose (99.35%, E. Merck, Mumbai, India) stock solution of concentration 27.75 mmol/L was prepared by dissolving required quantity in water. 4-AAP (4.92 mmol/L) solution was prepared by dissolving exact quantity in distilled water. IDB (30.72 mmol/L) solution was prepared by dissolving precisely weighed quantity in glacial acetic acid. All reagents used were of analytical grade and were used without further purification. Double distilled water was used throughout the experiment.



Scheme 1: Proposed mechanism for the formation of blue colored product

Apparatus

Kinetic and absorbance measurements were performed using a Jasco model UVIDEC-610 ultraviolet-visible spectrophotometer with 1.0 cm matched cells. The pH of the solution was measured using a pH - meter (model Equiptronics, Mumbai, India, and model EQ-614). Thermostatic water bath with shaker (model 206-88950-93, Shimadzu, Japan) was used to maintain the reaction temperature for color development.

Assay procedure

Quantification of hydrogen peroxide and POD

H_2O_2 was quantified in a 3 ml reaction solution containing 164.3 µmol/L 4-AAP, 102.4 µmol/L IDB, 4.73 nmol/L POD in 100 mmol/L KH_2PO_4/NaOH buffer of pH 6.3. The reaction was carried out at 23°C by adding 100 µL of different concentrations of H_2O_2 in the range of 1-255 µmol/L. The change in absorbance was monitored continuously at 620 nm against the corresponding control containing all reagents except H_2O_2. For the fixed time method, the reaction mixture was incubated for 5 min at 23°C, and the absorbance of the blue colored solution was measured at 620 nm.

POD was quantified in a 3 ml reaction mixture containing 164.3 µmol/L 4-AAP, 102.4 µmol/L IDB, 127 µmol/L H_2O_2 in 100 mmol/L KH_2PO_4/NaOH buffer of pH 6.3 with different concentrations of POD. The linearity of POD was quantified by both rate and fixed time methods.

Quantification of glucose

The glucose assay was carried out in a 3 ml reaction mixture containing 164.3 µmol/L 4-AAP, 102.4 µmol/L IDB, 9.46 nmol/L POD, 40 nmol/L GO_x in 100 mmol/L KH_2PO_4/NaOH buffer of pH 6.3 with varying the concentrations of glucose from 1 to 1184 µmol/L. The reaction was initiated at 23°C. The progress curve data was measured in 5 min against the corresponding control containing all the reagents except glucose. The initial rate of reaction was calculated from the time domain of 1 min at 620 nm. The calibration graph was obtained by plotting rate against the concentration of glucose. In fixed time method, the linearity of glucose was quantified by incubating the reaction mixture for 5 min at 23°C, and measuring the absorbance of the colored solution at 620 nm.

RESULTS AND DISCUSSION

Variables on the Determination of Glucose

The performance of bienzymatic reaction mainly depends on pH of the reaction mixture, temperature of the reaction condition and concentration of 4-AAP and IDB.

The effect of pH was studied using different buffer solutions such as citric acid/potassium citrate buffer (pH 3.6-5.6), potassium dihydrogen orthophosphate/sodium hydroxide buffer (pH 6.0-8.0), acetate/acetic acid buffer (pH 3.6-5.6), potassium dihydrogen orthophosphate/dipotassium hydrogen phosphate buffer (pH 6.0-7.5), and tris buffer (pH 8-11). The results presented in Figure 1 shows that the activity increased when the buffer pH was increased and reached maximum at pH 6.0. Hence, 100 mmol/L potassium dihydrogen orthophosphate/sodium hydroxide buffer of pH 6.0 was taken as the optimum for subsequent analysis.

Temperature sensitivity of the reaction mixture was studied from 10 to 80°C. The enzyme activity was measured as a function of absorbance of the colored solution. Increase in the temperature up to 23°C, increased the activity of the reaction. Above 23°C, the colored product intensity decreased; this can be attributed to a gradual decline in horseradish peroxidase activity due to heat inactivation as shown in Figure 2. Thus, the temperature was adjusted to 23°C for consequent analysis.

The effect of concentration of 4-AAP on the reaction rate was studied in the range of 10-657 µmol/L. Increasing the concentration of 4-AAP up to 164.3 µmol/L increased the rate of reaction, beyond which the reaction rate was independent of the concentration of 4-AAP. Hence, for further assays 164.3 µmol/L of 4-AAP was used. Similarly, the reaction rate increased with increasing concentration of IDB up to 102.4 µmol/L, beyond which the rate was constant. Hence, 102.4 µmol/L of IDB was selected as the optimum concentration for all further assays (data not shown).

Analytical Figures of Merits

The calibration graph for the quantification of H_2O_2 was established under the optimized conditions. The linear ranges obtained for the quantification of H_2O_2 were 1.95-63.8 µmol/L and 0.99-127.6 µmol/L by kinetic and fixed time methods, respectively, as shown in Figure 3. The apparent molar absorptivity of H_2O_2 assay was 1.03×10^4 L/mol/cm. The coefficient of variation was 1.9% for 31.9 µmol/L of H_2O_2 in 10 successive measurements. The detection and quantification limits were 0.45 µmol/L and 1.3 µmol/L, respectively. The linearity ranges obtained for the quantification of POD were 0.073-14.2 nmol/L and 0.14-9.46 nmol/L by kinetic and fixed time methods [Figure 4] with detection limits of 0.03 nmol/L (3δ) and 0.0017 nmol/L (10δ), respectively.

Figure 5 is a calibration graph for the quantification of glucose in the range of 1-1184 µmol/L. The calibration graph is linear in the range of 1.15-562 µmol/L and 2.31-370 µmol/L by kinetic and fixed time methods with a regression coefficient of 0.9982 and 0.9973, respectively. The apparent molar absorptivity for glucose was 0.7×10^4 L/mol/cm. The high molar extension coefficient indicates more stability of blue colored product. The limit of detection (LOD) and limit of quantification (LOQ) were 0.14 µmol/L and 0.44 µmol/L, respectively. The low values for LOD and LOQ indicate that

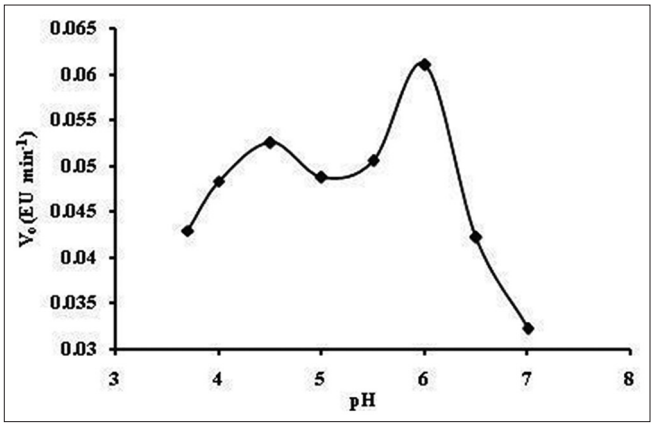


Figure 1: Effect of pH on reaction rate

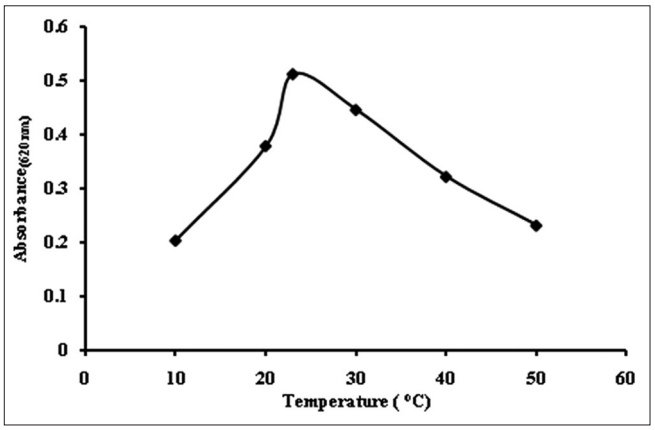


Figure 2: Effect of temperature on reaction rate

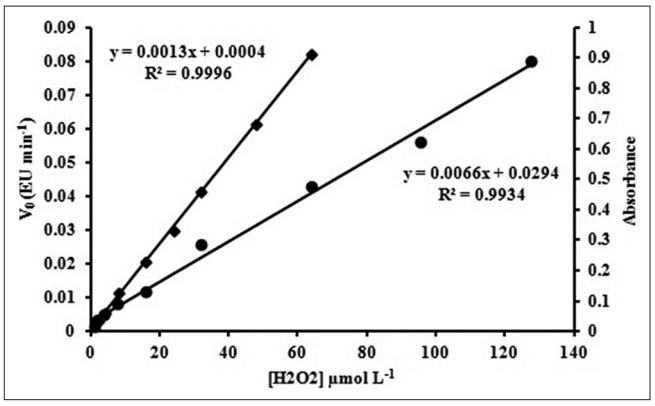


Figure 3: Calibration graph for the quantification of H_2O_2 by rate method (♦) and fixed time method (•)

the proposed method is highly sensitive, and these values are very much lower than the reported methods.[4,10,17] The coefficient of variation was 1.4% for the determination of 74 µmol/L glucose in 10 successive measurements.

Measurement of Michaelies Parameters on Enzymatic Reaction

Michaelies parameters for the glucose assay were studied under the optimized experimental conditions. A Lineweaver–Burk plot [Figure 6] was used for the evaluation of Michaelies–Menten constants for glucose concentration between 9.25 and 925 µmol/L. The linear regression equation of the straight line is $1/V = 2990.8/C_{glucose} + 8.5059$ with a correlation coefficient of 0.9967. The Michaelis–Menten constant for glucose was $K_m = 357$ µmol/L and maximum rate of reaction (V_{max}) was 0.117×10^{-6} mol/L/min. The low value of Michaelis–Menten constant indicates the existence of a stronger affinity between substrates and active site of enzyme and this value is relatively lower when compared to other methods[2,18,19] thereby indicating that the proposed method is highly sensitive, selective and specific. The catalytic efficiency (K_{eff}) and catalytic power (K_{pow}) of the proposed method were 0.0836×10^5 L/mol/min and $0.3 \times 10^{-3\ min-1}$, respectively.

Influence of Interfering Species

While establishing the glucose level in blood, foreign compounds in various amounts were added and their extent of interference was studied at glucose concentration of 0.148 mmol/L, and the results are presented in Table 1. The most potential interfering species are metal cations, saccharides, and reducing agents. Ascorbic acid is a strong reducing agent and it was found to interfere severely by H_2O_2 scavenging. Copper (II) has a strong inhibiting effect due to poisoning and inactivation of enzyme, cobalt (II) has a strong enhancing capacity due to catalytic activity on H_2O_2. As GO_x suffers a lack of absolute specificity it may undergo catalysis with galactose along with glucose in blood and show increased activity.[20] Saccharides also act as reducing agent and they can reduce H_2O_2 concentration[3] and also during the course of analysis disaccharides undergo hydrolysis or monosaccharides may undergo isomerization,[21] these two reactions produce glucose resulting in a positive error. To overcome the possible interference, blood sample should be diluted so that the concentration of interfering species gets lowered below the limits that would need for interference. The results revealed that most of the interfering species present in the blood samples contribute to minor extent in obtained glucose determination [Table 1]. Hence, the proposed method has good selectivity and specificity for glucose determination.

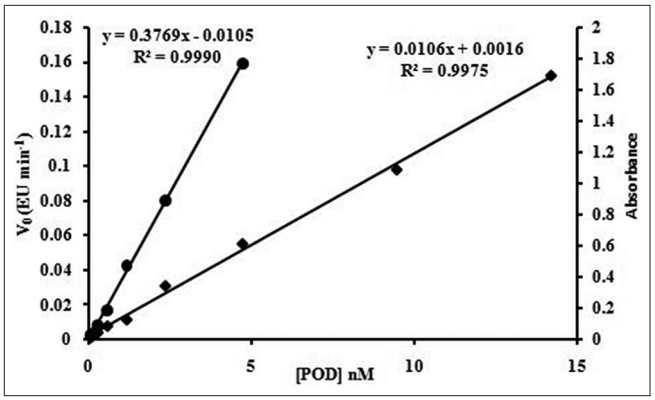


Figure 4: Calibration graph for the quantification of peroxidase by rate method (♦) and fixed time method (•)

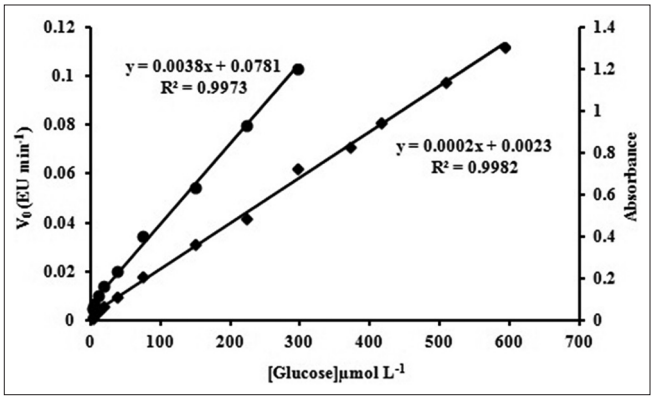


Figure 5: Calibration graph for quantification of glucose by rate method (♦) and fixed time method (•)

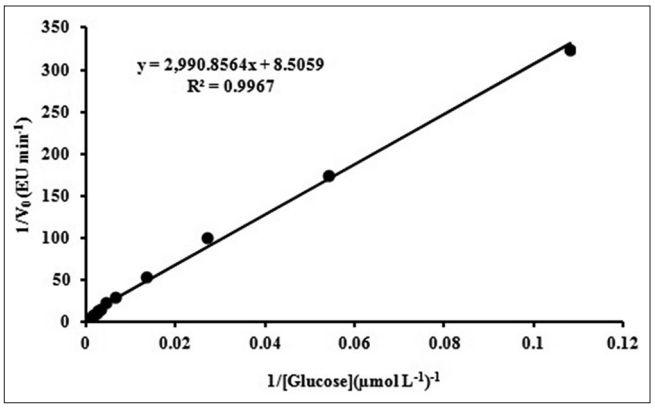


Figure 6: Lineweaver-Burk plot for glucose by the proposed method. The kinetic study was carried out with 40 nmol/L glucose oxidase

Accuracy and Precision

The accuracy and precision of the proposed method were studied using known amount of glucose within Beer's law range. The results are shown in Table 2. The inter-day precision was 0.53-2.9% (n = 10) and intra-day precision was 1.5-3.03% (n = 10). The accuracy range for glucose

Table 1: Influence of potential interfering species for the quantification of glucose

Interfering species	Concentration of interfering species in mmol/L	Tolerance ratio*
Ascorbic acid	0.0026	0.0179
Bilirubin	0.0048	0.0324
Iron (II), nitrite	0.0162	0.1095
Iron (III), L-tyrosine	0.1160	0.7838
F, L-tryptophan, L-cystein	0.297	2.01
Lactose, L-cystine, molybdenum	1.2173	8.22
L-Histidine, fructose, galactose	2.22	15.00
Isoleucine, D-asparagine, copper (II), magnesium (II), cobalt (II)	3.1763	21.46
EDTA, oxalic acid, uric acid chloride	5.702	38.53
Sucrose, maltose, magnesium (II), potassium	7.303	49.34
Citric acid, DL-methionine	8.732	59.00
Ammonium, sulfate	12.61	85.23
Carbonate, L-serine, creatinine	15.54	105.00
Fructose, D-galactose, zinc (II) mannose	18.50	125.01
Urea	36.075	243.75
Glycine, sodium	66.61	450.09
Acetone	790.6	5341

*Tolerance ratio correspond to the ratio of limit of interfering species concentration to that of concentration of glucose used.
EDTA: Ethylenediaminetetraacetic acid

Table 2: Inter-day and intra-day precision and accuracy ranges

Inter-day precision*			Accuracy range %	Intra-day precision*			Accuracy range %
Glucose mmol/L	SD (*n*=10)	RSD		Glucose mmol/L	SD (*n*=10)	RSD	
0.009	0.000183	2.9	91.32-94.78	0.009	0.000164	3.03	90.01-94.25
0.074	0.000243	1.08	94.21-98.54	0.074	0.000471	2.17	92.57-93.63
0.592	0.000634	0.53	93.23-101.54	0.592	0.001781	1.5	99.71-103.41

*Triplicate measurements. n: Number of measurements, SD: Standard deviation, RSD: Relative standard deviation

with concentration of 0.009, 0.074 and 0.592 mmol/L were 90-95%, 92-99% and 99-104%, respectively.

Quantification of Glucose in Human Serum Samples

To investigate the feasibility and reliability of the proposed method for quantification of glucose, five human serum samples were collected from a clinical laboratory. The blood samples were collected in heparinized tube, centrifuged and stored at −20°C. Necessary permission was obtained from Institutional Human Ethical Committee (IHEC-UOM no. 22/Ph.D/2008–09) of University of Mysore for the use of human blood samples in the experiment. The patients were well informed, and their consents were obtained before collecting the blood samples. The samples were analyzed by proposed method and also by reference enzymatic kit method.[22] The results obtained by these two methods are summarized in Table 3. The Bland–Altman plot [Figure 7] shows the relative difference between the proposed and reference enzymaic kit method. It gave significant constant bias of 0.07 mmol/L. The regression gave an intercept of 0.168 mmol/L and slope of 1.008 that was not considerably different from unity. The correlation coefficient was 0.9992 as shown in Figure 8 (95% confidence interval of 0.9965-0.9998) which implies that the proposed method is on equivalence with the enzymatic kit method.

The recovery study of human serum samples was also assessed by the standard addition method, and the results are shown in Table 4. The glucose recovery ranged from 91.92% to 108.5 % with a mean recovery of 100.43% which indicates that the proposed method is least affected by common interfering species present in human blood serum sample.

CONCLUSION

A novel and efficient bienzyme system for the quantification of glucose in human serum sample has been developed. No work has been reported for quantification of glucose in human serum sample based on the coupling of 4-AAP and IDB. The

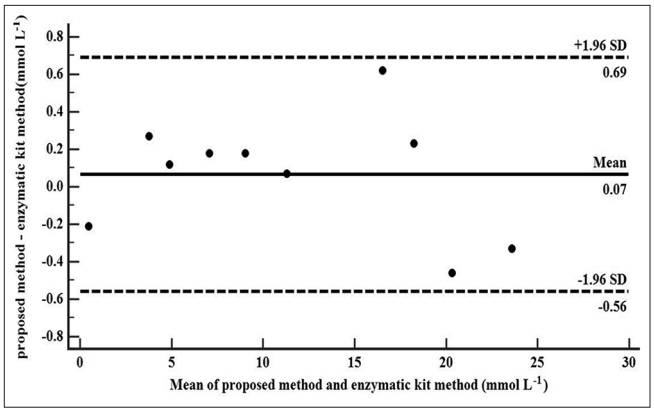


Figure 7: Bland–Altman analysis of glucose quantification in human serum samples using proposed method and by enzymatic kit method. The solid line is the bias that represents no difference between the human serum samples

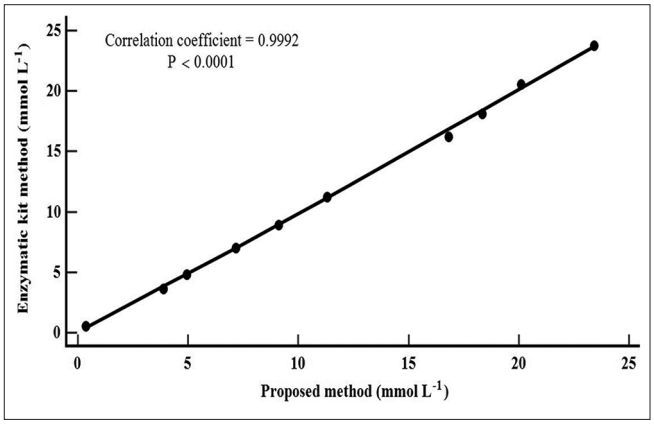


Figure 8: Comparison of results obtained by proposed method and enzymatic kit method for glucose quantification in human serum samples

small quantity of reagents needed, cost of these reagents are less when compared to other reagents, less reaction time (5 min) required and simple, inexpensive instrumentation used. The other advantage of the method is broad linearity range, high apparent molar absorptivity, lower detection limit, low Michaelies–Menten constant and relative standard deviation. The maximum wavelength (λ_{max}) of the colored product is 620 nm, which is out of the range of the absorption bands of bilirubin (380-530 nm) and ascorbic acid (300-400 nm).[23] Hence, this method has minimum interference by the bilirubin and ascorbic acid. The proposed method has excellent correlation with enzymatic kit method[22] with a correlation coefficient of 0.9992 and also having good recovery and high accuracy. Hence, the proposed method could be successfully adopted for quantification of glucose in clinical laboratories.

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Table 3: Determination of glucose in human serum samples

Samples	Proposed method[a] (mmol/L) (*n*=5)		Enzymatic kit method[b] (mmol/L) (*n*=5)	
	Found	RSD (%)	Found	RSD (%)
1	0.35	2	0.39	2.17
2	3.39	1.03	3.62	0.96
3	7.16	0.64	6.98	0.78
4	4.95	1.3	4.83	1.4
5	9.11	1.72	8.93	1.61

[a]Mean of five replicate determinations, [b]The samples were also analyzed in the laboratory by the enzyme kit method.[20] RSD: Relative standard deviation

Table 4: Recovery of glucose in human serum samples as analyzed by the proposed method

Samples	Proposed method[a] (mmol/L) (*n*=5)			Recovery (%, *n*=5)	RSD (%, *n*=5)
	Found	Added	Total		
1	0.35	0.5	0.88	108.5	1.27
2	3.89	1.15	5.0	98.97	0.74
3	7.16	2.31	9.56	101.26	1.59
4	4.95	4.62	9.15	91.92	0.94
5	9.11	3.52	12.86	102.52	0.84

[a]Mean of five replicate determinations. RSD: Relative standard deviation

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