*Indian Journal of Clinical Biochemistry, 2009 / 24 (1) 36-41*

# ESTIMATION OF PLASMA HAEMOGLOBIN BY A MODIFIED KINETIC METHOD USING O-TOLIDINE

**Madhur M Goyal and Anjan Basak**

*Department of Biochemistry, Jawaharlal Nehru Medical College, Wardha-442004, India.*

**ABSTRACT**

*Measurement of plasma hemoglobin is useful in variety of clinical conditions. In the present study we have developed a kinetic method to estimate plasma haemoglobin by using o-tolidine. This method is sensitive, rapid, economical, simple and less influenced by interfering substances. It measures plasma haemoglobin in the range of 6 to 400 mg/L (normal range < 50 mg/L) in less than two minutes and can be easily automated.*

**KEY WORDS**

*Plasma hemoglobin, o-Tolidine, Kinetic method.*

## Introduction

Benzidine test is still being used to detect haemoglobin (blood) qualitatively in biological fluids. As benzidine is carcinogenic (1), it was recommended to replace it by its non-carcinogenic and less toxic derivatives- o-tolidine and tetramethylebenzidine (TMB) (2).

Workers has also developed quantitative test to measure plasma haemoglobin using different substrates, like, TMB (3), 2,2'-Azino-di(3- ethylbenzthiazoline-6-sulfonic acid) ABTS (4), Chlorpromazine (5) etc. These methods are based on the principle that the catalytic action of haem containing proteins on hydrogen peroxide produces oxidizing species, which react with substrate to produce a color change.

We followed the same principle and developed a new method to estimate plasma haemoglobin using o-tolidine. In present method reagents employed, are very economic than TMB and other substrates exploited in various studies (3, 4, 5, 6). Our method measures plasma haemoglobin in micromole concentration ranges from 6 to 400 mg/L (10 mg/L=0.16 µmol/L) with precision, acceptable linearity, reproducibility. This method is sensitive and range of measurement is wider than other methods (4, 5, 7). In this method we measure the change in absorbance per minute with delay time 30 sec. at 630 nm to compare sample with standards. This approach makes our method rapid and less influenced by interfering substances unlike other methods (4,5,8,9). Furthermore it uses comparative less toxic reagent than KCN (10) and does not require any specialized instrument like centrifugal analyzer (8).

***Address for Correspondence :***

**Prof. Anjan Basak,**
Department of Biochemistry,
Jawaharlal Nehru Medical College,
Wardha-442004
E-mail: drabasak1@yahoo.com

## MATERIALS AND METHODS

**Principle:** Haemoglobin (or haem) acts as chemical catalyst to breakdown hydrogen peroxide into water and nascent oxygen. Nascent oxygen oxidises o-tolidine to give oxidized product which is of green blue colour. Rate of colour development is measured at 630nm which is directly proportional to haemoglobin concentration.

**Solvent:** 20 ml of glacial acetic acid (GAA) was mixed with 80 ml of ethanol and used for preparation of stock and working solutions.

**o-Tolidine stock solution:** Dissolve 2 gm of o-tolidine in 100 mL of solvent to make a stock solution. (stable for 1-2 months when stored in a brown bottle at 4°C). Dilute one volume of the stock solution 5 fold with the same solvent to make the working reagent (0.4 gm/dL). 100 µL Triton-X-100 was mixed with 100 ml of working reagent to increase the linearity of kinetic reaction. This reagent is stable for one day at room temperature. It should be centrifuged if working reagent is not transparent.

**Hydrogen peroxide solution:** Prepare 2% (v/v) hydrogen peroxide solution in analytical water and add 2.26 gm sodium acetate (in 100 ml) to create a buffering environment with GAA present in final reaction mixture. With this buffer pH is maintained between 3.0 to 3.5 in the final reaction mixture. This solution can be used for 6 to 8 hrs.

**Hemoglobin standard solution**: It was prepared as described by Levinson et al. (8). In brief, Wash 1 mL of packed erythrocytes twice with 2-mL of isotonic saline. Then haemolyze them by diluting with equal amount of water and freezing at -20 °C. Thaw this hemolysate at room temperature, centrifuge (4000g, 15 min), and estimate the hemoglobin in the supernatant by the cyanmethaemoglobin method (11). This haemoglobin solution is stable for at least three weeks at 4°C. Dilute it to yield hemoglobin standards with concentrations ranging from 6 to 400 mg/L, for preparation of standard.

**Effect of bilirubin**: 50 mg of bilirubin was dissolved in 10 mL of 0.1 mol/L sodium carbonate and diluted to obtain the desired concentrations of bilirubin.

**Hemoglobin assay procedure**: Pipete 1.0 ml of working solution and 1 mL of $H_2O_2$ solution into test tubes, mix well and wait for 5 min for maturation of reagent. Add 10 µL of each hemoglobin standard and sample into respective test tubes, mix well and immediately measure ΔAbs per minute at 630 nm with a delay time of 30 secs. Analytical grade water is to be used as reagent blank.

For the new procedure any spectrophotometer capable of reading an absorbance of 0.001 units at 630 nm and a wavelength accuracy of 2 nm can be used. Cuvettes with a 1-cm light path are preferred.

## RESULTS AND DISCUSSION

Initially, we started the work for comparison of sensitivity between benzidine and o-tolidine in qualitative assay of haemoglobin and found same results as reported (2), like, o-tolidine is more sensitive than benzidine and absorbance peak of o-tolidine reaction is obtained at 450 nm. o-tolidine reaction was performed in GAA and ethanol separately and we observed that in GAA, colour develops immediately (comparatively slower in ethanol) and after few minutes it get disappear. It is one of the few inherent limitations of methods with benzidine derivatives as chromogens. Other limitation is due to the presence of heme containing proteins like cytochrome, peroxidase and catalase, which gives false higher values. To overcome these limitations, we scrutinize few modifications with o-tolidine reaction, are discussed hereunder.

**Effect of integration of GAA and ethanol:** In a solution of GAA and ethanol (20:80), the rate of reaction decreases and absorption maximum ($\lambda_{max}$) reallocate from 450nm to 630 nm (Figure 1). The reason behind it is discussed afterward. By this shifting this method became less influenced by many interfering substances like bilirubin, which gives a prominent peak at 453 nm (405-493nm) (9). In our graph we found an

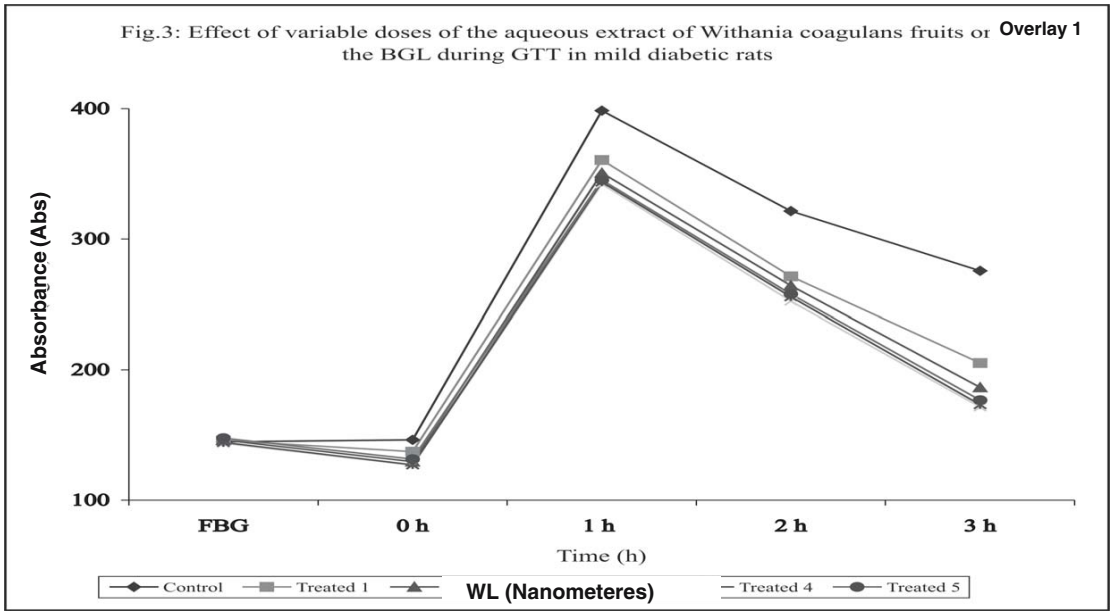


**Fig. 1: Wavelength scan of o-tolidine reaction in solvent.**

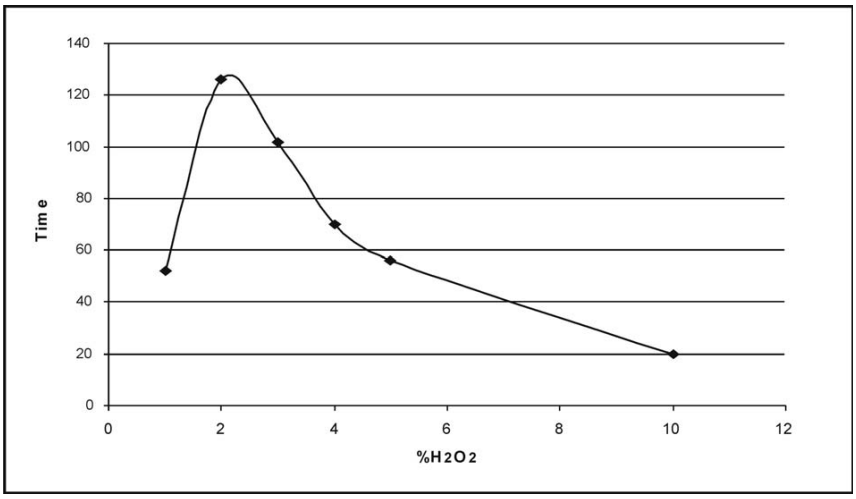


**Fig 2: Effect of varying concentration of $H_2O_2$ on reaction linearity**

extra peak at 640 nm which origin is not known to us.

**Effect of different $H_2O_2$ concentration:** We found that with 2% (v/v) $H_2O_2$ solution, o-tolidine reaction showed a linear increase in absorbance upto more than 120 seconds (Figure 2). Higher concentration of $H_2O_2$ made reaction faster and was completed within few seconds. Whereas at low concentration (<2.0%), $H_2O_2$ itself became a rate limiting factor and bring about end of reaction.

**Effect of buffering environment**: The presence of sodium acetate and GAA in reaction mixture creates buffer and increase the change of absorbance at 630 nm (Figure 3). The solvent and the buffer environment make intermediates of reaction more stable so that $\lambda_{max}$ shifted from 450nm to 630 nm. This conclusion is supported by two previous findings by I) Stanley et al (8) reported that if the concentration of acetic acid in the reaction mixture decreases to <100 mL/L, the solution becomes unstable and turns blue. II) David et al (12) showed mechanism of oxidation of benzidine derivative (TMB) by which two type of intermediate are generated in reaction mixture and they are stable at acidic pH. The same may happen in o-tolidine reaction, as TMB is dimethyl derivative of o-tolidine.

The presence of sodium acetate and GAA in reaction mixture readjusts pH of solution. It might be the explanation of maturation of reagent, otherwise not known to us.

**Reagent blank:** We observed that there is a substantial change in absorbance in reaction mixture with prolong time, even when no sample is added. It does cause poorer reproducibility in the low hemoglobin range. As this assay is based on change in absorbance, we used analytical grade

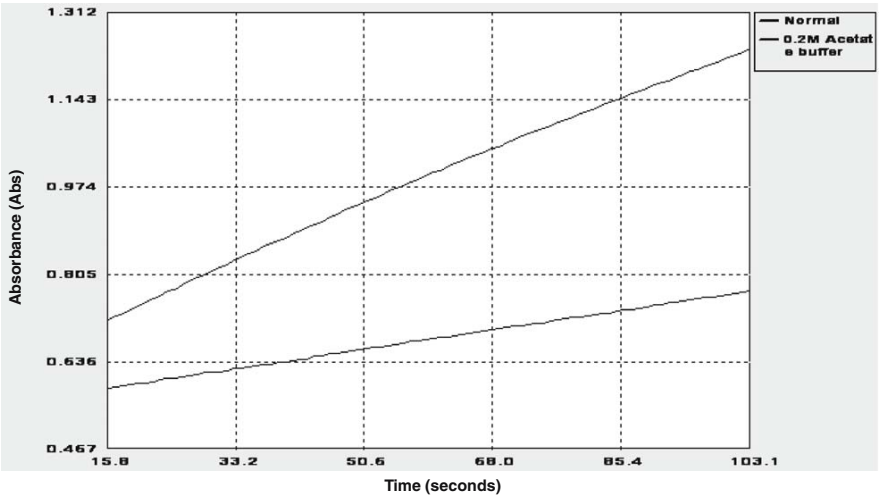


**Fig 3: Effect of buffering environment on rate of reaction at 630 nm (upper line is with buffer environment)**

water as reagent blank.

**Standard curve and linearity**: As shown in Figure 4, a good linear relation with ΔAbs was obtained for hemoglobin concentrations between 6 and 400 mg/L. Reproducibility was examined by 10 replicate assays at different plasma hemoglobin concentrations (data not shown). These results are within the 2 SD precisions for the assay.

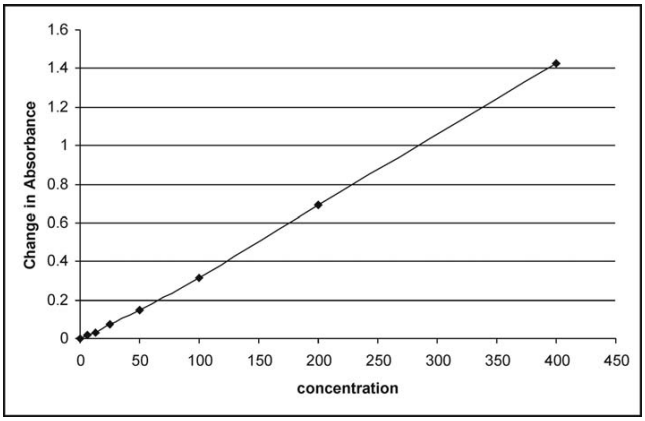


**Fig 4: Standard graph is plotted with ΔAbs per minute (with delay time of 30 sec) against different concentration of haemoglobin (mg/L)**

**Effect of ascorbic acid:** Among water-soluble antioxidants, ascorbic acid plays the key role in plasma with normal range upto 1.5 mg/dl (13). It can interfere in oxidation of o-tolidine. Figure 5 shows that there is no effect of increasing concentration of ascorbic acid in sample. It is due to presence of acetic acid (14).

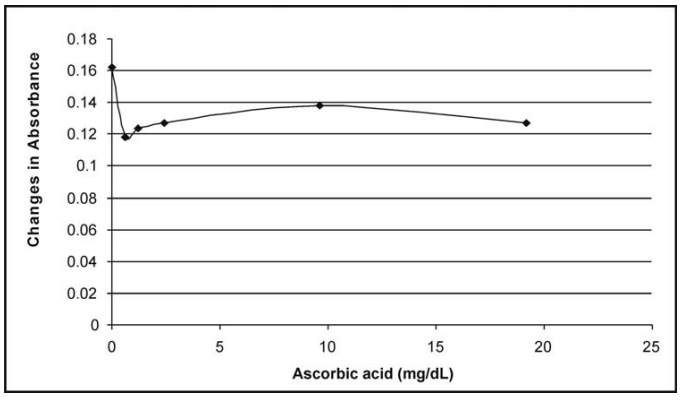


**Fig 5: Effect of ascorbic acid**

**Effect of glucose:** Haemoglobin solutions (25mg/L) were prepared with different glucose concentrations. Glucose has no interference in our method upto 600 mg/dL (Figure 6).

**Effect of Bilirubin:** Bilirubin was added to give final concentrations of 0.62, 1.25, 2.5, 5.0, 10.0 and 20.0 mg/dL in sample with hemoglobin concentration 12 mg/L (Figure 7). At

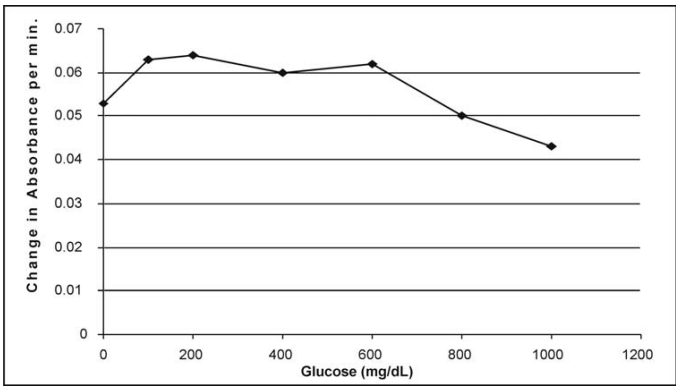


**Fig 6: Effect of glucose**

lowest (0.62 mg/dL) concentration, bilirubin did not affect the reaction. Analytical recovery of hemoglobin in next three concentrations (upto 5 mg/dL) was between 99.16 and 105.8%. More than 5 mg/dL bilirubin inhibited the o-tolidine reaction.

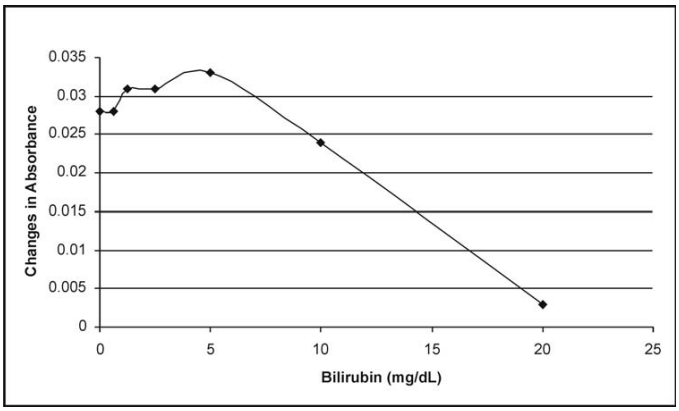


**Fig 7: Effect of bilirubin**

**Effect of heat on peroxidase and haemoglobin**: Most of the quantitative methods for estimation of haemoglobin are end point (4, 8). Their incubation time vary from 3 to 90 mins. (4,5,8) Heme containing protein like peroxidase gives false positive values in these methods (2). This modified method is not influenced by normal activity of plasma peroxidase, so we used peroxidase with increased activity (16 U/ml). It was found equivalent to 50 mg/L haemoglobin. When sample was kept in boiling water bath for 2 min and 5 min., peroxidase activity decreased and lost in five min (Figure 8) whereas haemoglobin was not affected (Figure 9). So if increased activity of peroxidase is suspected in sample it should be placed in boiling water bath for 5 mins.

To obtain good results every effort must be made to prevent haemolysis during the collection, transport and storage of the blood. It is recommended to collect blood either in heparin or

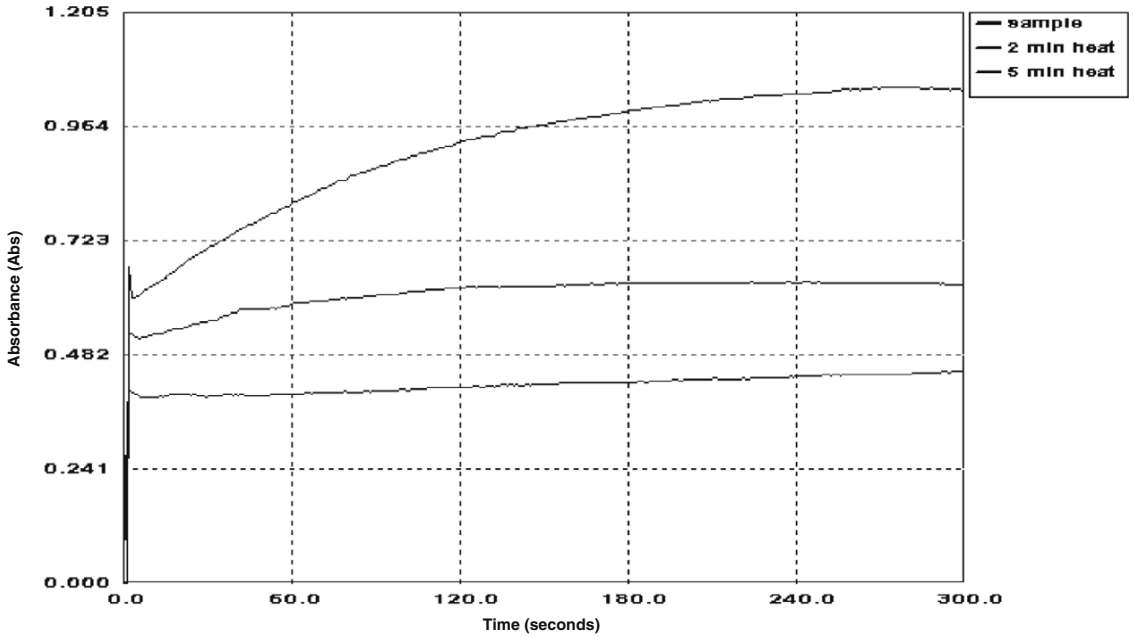


**Fig 8: Effect of heat on activity of peroxidase. Time scan is recorded upto 5min. upper most scan is obtained from peroxidase (16 U/L). Activity is decreased after 2 min boiling (middle line) and almost lost in 5 min (lowest line)**

EDTA. Because fHb is increased during clotting, serum should not be used (15).

The reference interval for plasma haemoglobin is usually given as 0-50 mg/L (4,5,8,10,11) although values < 100 mg/L are rarely clinically important (8,9). The use of plasma haemoglobin assay in stored blood for transfusion and haemolytic events arising from disseminated intravascular haemolysis or artificial heart valves can help in rapid corrective measure, if needed (16). Other haemolytic conditions with substantial intravascular haemolysis includes paroxysmal nocturnal hemoglobinuria (PNH), sickle-cell disease (SCD), thalassemias, hereditary spherocytosis and stomatocytosis, microangiopathic hemolytic anemias, pyruvate kinase deficiency, ABO mismatch transfusion reaction, paroxysmal cold hemoglobinuria, severe idiopathic autoimmune hemolytic anemia, infection-induced anemia, malaria, cardiopulmonary bypass, mechanical heart valve-induced anemia and chemical-induced anemias (17). In these conditions hemoglobin is released from the erythrocyte and when the capacities of protective hemoglobin-scavenging

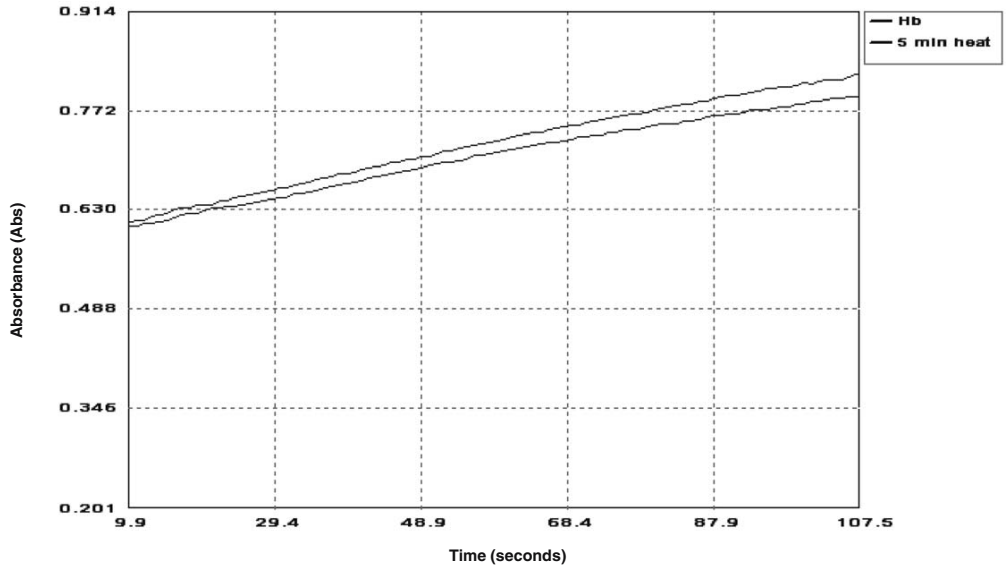


**Fig 9: Effect of heat on haemoglobin. ΔAbs. (between 30 s & 90 s) is almost same without heating (upper line) and after 5 minute heating (lower line)**

mechanisms have been saturated, levels of cell-free hemoglobin increase in plasma.

In conclusion, we consider the modifications done by us in o-tolidine reaction is helpful to develop a simple, sensitive, reliable method that is economic as well as easy to run on automated analyzers without much influence of common interfering substances.

**ACKNOWLEDGEMENT**

The authors highly appreciate Datta Meghe Institute of Medical Sciences University, Wardha, for funding this work.

**REFERENCES**

1. Spitz S, Maguigan WH, Dobriner K. The carcinogenic action of benzidine. Cancer 1950; 789-804.
2. Varley H. Practical Clinical Biochemistry 4th Ed., CBS publishers & distributors,Delhi, 1969: P.344-8.
3. Pillier-Loriette C, Wingerter P, Gerota J, Schenmetzler C, Determination of plasma hemoglobin in fresh plasma for therapeutic use by the TMB (3,3',5,5'-tetramethyl benzidine) method. Rev Fr Transfus Immunohematol. 1983; 26(3): 299-312.
4. Takayanagl M,Yashiro T. Colorimetry of Hemoglobin in Plasma with 2,2'-Azino-di(3- ethylbenzthiazoline-6-sulfonic acid) (ABTS). Clin Chem 1984; 30(3): 357-9.
5. Chalmers AH, Snell LE. Estimation of Plasma and Urinary Hemoglobin by a Rate Spectrophotometric Method. Clin Chem 1993; 39 (8): 1679-82.
6. Lee KT, Ling AM. Determination of micro-quantities of haemoglobin in serum. Microchimica Acta 1969 ; 57(5) : 995-1002.
7. Lijana RC, Williams MC. Tetramethylbenzidine—a substitute for benzidine in hemoglobin analysis. J Lab Clin Med 1979; 94(2): 266-76.
8. Levinson SS, Goldman J. Measuring hemoglobin in plasma by reaction with tetramethylbenzidine. Clin Chem 1982; 28(3): 471-4.
9. Fairbanks VF, Ziesmer SC, Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. Clin Chem 1992; 38(1): 132-40.
10. Kruszyna R, Smith RP, Ou LC. Method for measuring increased plasma hemoglobin in the presence of erythrocytes. Clin Chem 1977; 23(11): 2156-9.
11. Dacie JV, Lewis SM. Estimation of plasma Haemoglobin, Practical Haematology 6th Ed.,Churchill Livingstone,London, 1984: P. 139-40.
12. Josephy PD, Eling T, Mason RP.The Horseradish Peroxidase-catalyzed Oxidation of 3,5,3',5'-Tetramethylbenzidine . Free radical and charge-transfer complex intermediates. J Biol Chem 1982; 257(7): 3669-75.
13. Burtis CA, Ashwood ER, Bruns DE,editors. Tietz textbook of clinical chemistry and molecular diagnosis, 4th ed. New Delhi: Elsevier, 2006:2302pp.
14. Henery. Clinical chemistry-principals and techniques, Chapter 21, subsection "Urine", 1964: P. 779-82.
15. Unger J, Fillipi G, Patsch W. Measurement of free haemoglobin and haemolysis index: EDTA or lithium-heparinate plasma? Clin Chem 2007; 53(9): 1717-18.
16. Wood WG, Kress M, Meissner D, Hanke R, Reinauer H. The Determination of free and protein-bound haemoglobin in plasma using a combination of HPLC and absorption spectrometry. Clin Lab 2001; 47: 279-88.
17. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin.A novel mechanism of human disease. JAMA 2005; 293(13): 1653-62.