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Plasma Haemoglobin Determination Using Chlorpromazine as a Non-Carcinogenic Reagent

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Summary: Plasma haemoglobin was assayed with the non-carcinogenic reagent phenothiazine. This method is sensitive and allows the measurement of plasma haemoglobin concentrations in the range 4–500 mg/l with a within-run CV of 2.1%, and a between-run CV of 4.3%. A spectrophotometric scanning method (x) based on the determination of haemoglobin as haemiglobin cyanide using the Soret band at 419 nm correlated well with the phenothiazine method (y): $y = 1.07x + 15.8$, $r = 0.995$, $n = 31$. It was found that the absorbances in the phenothiazine method were markedly dependent on the concentration of phosphoric acid.

Introduction

The quantitative determination of plasma haemoglobin is of clinical importance in haemolytic disorders, which occur either in vivo (1, 2) or in vitro (3). However, the carcinogenity of many commonly used reagents is undesirable for routine laboratories. Benzidine, *o*-tolidine (4) and dicarboxidine (5), used in previous studies, are all carcinogens. Of the suggested alternative non-carcinogenic chromogens, tetramethylbenzidine (6, 7) aminophenazone (8), and 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (9, 10) have proved suitable for the quantitative determination of plasma haemoglobin. The reaction of these materials with haemoglobin is based on the peroxidase activity of the haemoprotein. Spectrophotometric scanning techniques do not require carcinogenic reagents (11, 12, 13), but are only accessible to those laboratories possessing scanning equipment.

Recently, *Ferencz & Bacso* (14) developed a method for the determination of haemoglobin in plasma or serum using phenothiazines. This method is also based on the peroxidase activity of haemoglobin, but the main reason for recommending this reagent is its non-carcinogenity. They also introduced the use of phosphoric acid to stabilize the coloured reaction end product. We have evaluated a modification of the method using chlorpromazine and found it sufficiently rapid and sensitive to measure plasma haemoglobin concentrations of 4–500 mg/l.

Materials and Methods

Glacial acetic acid, phosphoric acid and Na_2EDTA were from E. Merck (Darmstadt, F. R. G.). Chlorpromazine (250 mg) (Ph. Nord., Yliopiston Apteekki, Helsinki, Finland) was dissolved in 100 ml of 5.4 mmol/l Na_2EDTA. This solution is stable for one week at + 4 °C. Phosphoric acid was diluted to 5 mol/l with distilled water. Hydrogen peroxide (Bang & Co., Helsinki, Finland) solution was freshly prepared daily from 300 g/kg H_2O_2 by diluting 3.4 ml of H_2O_2 in 100 ml of distilled water. All reagents were of "pro analysis" quality. A 300 µl aliquot of whole blood, whose haemoglobin concentration had previously been determined by Coulter Counter® S-PLUS IV (Coulter Electronics, Inc., Hialcah, U. S. A.) using the haemiglobin cyanide technique checked by Coulter quality control 4c, was haemolyzed by diluting to 100 ml with water. This solution was used as a haemoglobin standard and is stable at 20 °C for at least 6 months. Patient plasma samples were collected using either sodium heparin or potassium EDTA tubes (Venoject, Terumo Corp., Tokyo, Japan) which are used routinely in our laboratory.

To 20 µl of plasma or standard we added 0.5 ml of chlorpromazine solution, 1.4 ml acetic acid and 0.2 ml of 5 mol/l H_3PO_4. Reaction was started by adding 1.0 ml of H_2O_2. The final concentrations of the reagents of the assay mixture were:
chlorpromazine 0.4 g/l,
acetic acid 7.9 mol/l,
H_3PO_4 0.32 mol/l and
H_2O_2 0.09 mol/l.

The absorbance was measured against a blank which had been prepared using pooled haemoglobin-free plasma. The absorbance readings at 528 nm (Zeiss PM 2 DL Spektrophotometer, Zeiss, Oberkochen, F. R. G.) were recorded exactly eight minutes after the addition of the H_2O_2 solution.

The haemoglobin-free plasma used as a blank was checked by measuring the absorbance of the reaction against the reaction of the sample replaced by water. This blank absorbance reading was not allowed to exceed 0.07.

The effects of bilirubin and ascorbic acid on the assay were studied. Bilirubin (Fluka AG, Buchs, Switzerland) 100 mg, was dissolved in 0.1 mol/l NaOH and diluted to 20 ml with 50 g/l of bovine albumin solution (Sigma, St. Louis, USA). Ascorbic acid (Merck) 200 mg was dissolved in 100 ml of water. These stock solutions were added to pooled plasma.

Results and Discussion

The linearity of the method was checked by making a series of dilutions of the standard in water and analysing as described. To confirm the standardisation of the method we used a haemoglobin control from Merz + Dade AG (Duedingen, Switzerland, haemoglobin concentration 110 g/l). The control was diluted and used in the same way as the standard. Figure 1 shows the results obtained using dilutions of the standard and the control.

The within-run coefficient of variation (CV) was 2.1% (n = 10) using a patient sample containing 362 mg/l haemoglobin. The between run CV was 4.3% (n = 10, sample containing 251 mg/l haemoglobin). The analyses for determining the between run variation were performed during a seven-week period.

It was found that a final concentration of 0.4 g/l chlorpromazine in the assay mixture was sufficient, provided the chlorpromazine solution was freshly prepared. Higher concentrations of chlorpromazine led to absorbance readings that were too high for measurement with the spectrophotometer at the plasma haemoglobin level of 430 mg/l and above. During storage the chlorpromazine solution slightly looses its capacity to be peroxidized by hydrogen peroxide, and the intensity of the coloured end product as well as the linearity of the method are decreased.

The concentration of phosphoric acid in the reaction mixture was of critical importance. The absorbance of the reaction end product was found to increase as the concentration of the phosphate increased (fig. 2). However, the absorbance maximum was reached later in higher phosphate concentrations and the colour was more unstable (fig. 2). *Ferencz & Bacso* (14) used the final concentration of 0.16 mol/l H_3PO_4 in the assay mixture to stabilize the colour of the end product. However we found that 0.16 mol/l instead of 0.32 mol/l H_3PO_4 in the reaction mixture caused a dramatic

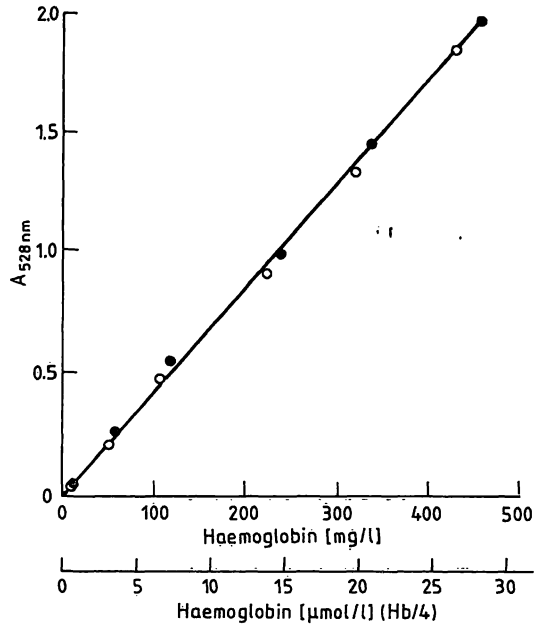

Fig. 1. Linearity of absorbance readings against haemoglobin concentration;
(●), (○) represent dilutions of the standard and control, respectively.

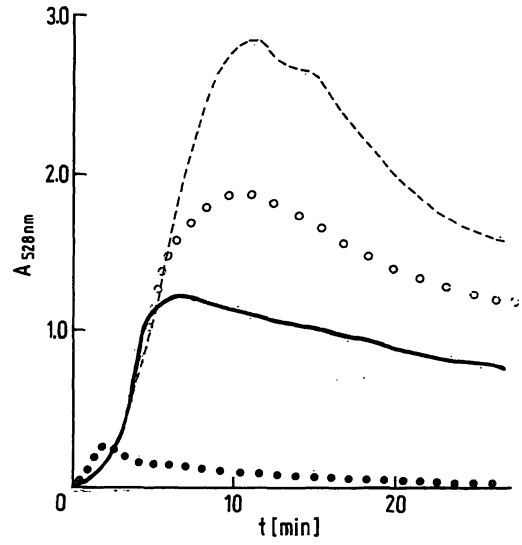

Fig. 2. Effect of the concentration of H_3PO_4 on the colour development in the reaction mixture (chlorpromazine 0.4 g/l, glacial acetic acid 7.9 mol/l, H_2O_2 0.09 mol/l and 20 µl of a sample with 380 mg/l of haemoglobin);
(●●●) without H_3PO_4,
(——) 0.32 mol/l H_3PO_4,
(○○○) 0.64 mol/l H_3PO_4,
(- - -) 0.95 mol/l H_3PO_4.
The absorbance readings were taken at a 30 seconds interval.

reduction in the absorbance values, and the linearity of the method decreased (fig. 3). A final concentration of 0.32 mol/l H_3PO_4 proved to be the optimal with respect to absorbance and stability for plasma haemoglobin concentrations of 4–500 mg/l. Samples with haemoglobin concentrations above 500 mg/l were diluted 1 : 5 with 9 g/l NaCl.

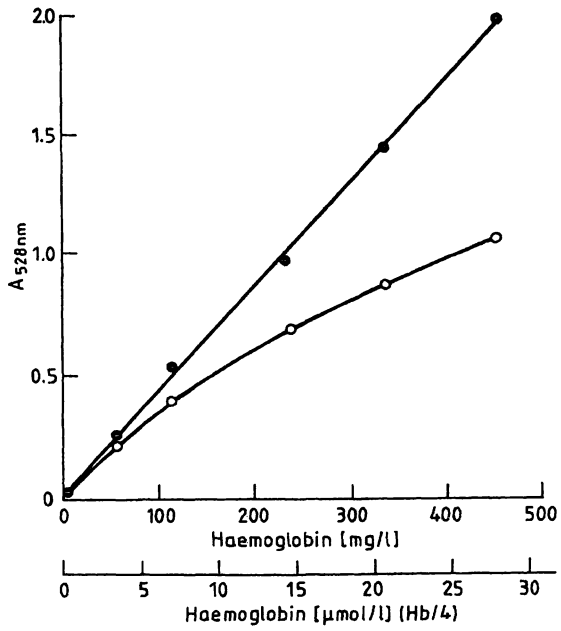

Fig. 3. Effect of the concentration of H_3PO_4 in the reaction mixture.
(●), (○) represent the absorbances against concentration when the reaction mixture contained 0.32 mol/l or 0.16 mol/l H_3PO_4, respectively.

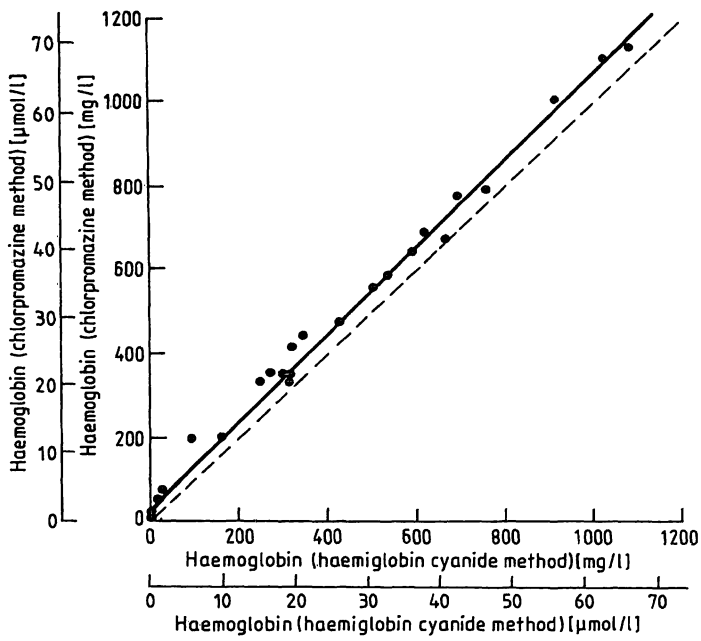

Fig. 4. Comparison of results observed using the chlorpromazine and haemiglobin cyanide method, dotted line: $y = x$.
Cf. text for details.

The detection limit of the assay was 4 mg/l which corresponds to an absorbance reading of 0.01 at 528 nm. The normal range for plasma haemoglobin is usually given as 0–25 mg/l (4, 7) or 0–50 mg/l (8, 10).

The chlorpromazine method described (y) was compared with the spectrophotometric scanning method (x) based on the determination of haemoglobin as haemiglobin cyanide using the Soret band at 419 nm (4). A linear regression equation for the two methods was $y = 1.07x + 15.8$ (31 patient samples), with haemoglobin concentrations between 0–1148 mg/l ($r = 0.995$, fig. 4). The chlorpromazine method gave slightly higher results (paired t-test $p < 0.001$).

Lipaemic samples may cause erroneously high results. For turbid samples we prepared sample blanks: 20 µl of the sample was first treated with 1 ml of H_2O_2 for 10 minutes, followed by addition of other reagents.

Previous studies have demonstrated that the anticoagulants oxalate, citrate, EDTA and heparin have no effect on the phenothiazine methods. Ascorbic acid in high concentration does inhibit the colour development of both the phenothiazine and the benzidine method in plasma and in urine (14, 15). We tested the effect of ascorbic acid and bilirubin on the chlorpromazine method. Ascorbic acid was added to plasma to give final concentrations of 32, 56, 111 and 222 µmol/l; and bilirubin was added to plasma to give concentrations of 40, 80, 160 and 388 µmol/l with a haemoglobin concentration of 636 mg/l. Analytical recoveries of haemoglobin in these samples were 96%–100% for plasma spiked with ascorbic acid and 102%–109% for plasma spiked with bilirubin. We conclude that neither ascorbic acid nor bilirubin at their physiological or elevated concentrations have any marked effect on this method.

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