A Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma and Urine

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In various pathologic conditions, but especially in hemolytic disease, it is of interest and importance to measure accurately the concentration of dissolved hemoglobin in the urine and plasma. This is easy to do when the concentration of Hb is high so that standard methods of hemoglobinometry[4] can be adapted to the purpose. With low concentrations or small volumes of material it is necessary to amplify the photometric quality of the pigment. This may be done variously. Flink and Watson[5] proposed the use of pyridine, but this method is not accurate for dilute Hb solutions. The benzidine reaction has been the most widely used. It was first proposed by Wu[10] as a micromethod for hemoglobinometry and, although the method has been repeatedly modified,[1, 3, 7-9] it has not proved to be an accurate method for measuring hemoglobin in the plasma[2] or the urine.[6]

We have studied the benzidine method and have found in plasma and urine several sources of interference with the color development of the benzidine-hemochromogen reaction. In plasma there is an interfering substance, perhaps lipoprotein, that can be dissolved in a mixture of methanol and chloroform. The addition of alcohol to the reaction, as proposed by Ham,[7] improves the recovery from plasma, but the recovery is not complete and it varies with the amount of plasma that is used, a source of inconvenience when it is necessary to dilute the unknown in order to bring it within the range of the method. Alcohol also causes darkening of most benzidine solutions so that it is usually necessary to make the reagent fresh each time it is to be used or to add the alcohol to each reaction after the hemoglobin and benzidine solution have been mixed. We have found this latter expedient a useful one. In our clinical studies for the past two years, we have used a modification of Ham's method, mixing 0.02 ml. of the unknown with 2 ml. of a 2 per cent solution of benzidine dihydrochloride in 20 per cent acetic acid and then adding 1 ml. of 95 per cent ethanol before the addition of 1 ml. of 0.5 per cent hydrogen peroxide. Even with this method the recovery of hemoglobin in the presence of plasma may be deficient by as much as 10 to 15 per cent (table 1).

Urine contains urates, sulfates and perhaps other materials that form insoluble complexes with benzidine and not only interfere with color development but also cause turbidity. Since the degree of interference is proportional to the concentration of these materials it is not satisfactory to balance the reaction of a urinary unknown by adding a similar amount of normal urine to the known standard hemoglobin solution.[6]

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TABLE 1.—*Inhibition of the Benzidine Reaction by Plasma*

In the presence of plasma there was only 40 per cent recovery (tubes 1 vs. 4). With the addition of alcohol there was 85 per cent recovery (tubes 2 vs. 3). By increasing the amount of alcohol it is possible to achieve greater than 100 per cent recovery, which suggests that the effect of alcohol is not specific.

	Tube No.				
	1	2	3	4	5
Benzidine-2 HCl Solution, 2% (ml.)	—	—	—	—	2
B-2 HCl plus Hb Standard (ml.)	2	2	2	2	—
Plasma (ml.)	0.02	0.02	—	—	0.02
Ethanol 95% (ml.)	—	1	1	—	1
H_2O_2, 0.5% (ml.)	1	1	1	1	1
Acetic acid 20% (ml.)	10	9	9	10	9
Result (Optical Density)	0.06	0.29	0.335	0.14	0.01

TABLE 2.—*Lack of Plasma Interference with the Proposed Method*

Using the proposed modification the recovery hemoglobin in this trial was slightly better than 98 per cent when D of 2 is compared with the sum of 1 plus 3.

	Tube No.		
	1	2	3
Benzidine Base Solution, 1% (ml.)	—	—	1
BBS plus Hb Standard (ml.)	1	1	—
Plasma (ml.)	—	0.02	0.02
H_2O_2, 0.5% (ml.)	1	1	1
Acetic acid, 10% (ml.)	10	10	10
Result (Optical Density)	0.310	0.317	0.013

With these problems in mind a method has been developed that improves the accuracy and the usefulness of the benzidine method of microhemoglobinometry. Recovery exceeds 95 per cent (table 2).

MATERIALS AND METHODS

Reagents. (Glassware used for preparation and storage should be acid cleaned. Check the water used for reagents with $BaCl_2$ to be sure that it is free of sulfate.)

1. Benzidine reagent, 1 per cent. Dissolve 1 Gm. of benzidine base in 90 ml. of glacial acetic acid and make up to 100 ml. with distilled water. The reagent should be kept in the refrigerator, and the addition of the small amount of water prevents its solidifying at ordinary refrigerator temperatures.

2. Hydrogen peroxide, 1.0 per cent. Care should be taken to avoid peroxide that has been stabilized with sulfuric acid because sulfate precipitates benzidine. Prepare the peroxide solution fresh every several days. It should be refrigerated when not in use.

3. Standard hemoglobin solution. Wash 2 ml. red cells and lyse them by freezing and thawing. With 0.9 per cent NaCl solution dilute the Hb to approximately 10 Gm. per 100 ml. and determine its exact concentration by ordinary clinical hemoglobinometry.[4] Prepare the standard by diluting 0.02 ml. (Sahli) of this concentrated Hb solution in 10 ml. of saline. The standard contains 20 mg. Hb per 100 ml. if the concentrated solution has 10 Gm. per 100 ml.

4. Diluent consists of 10 per cent glacial acetic acid in sulfate-free distilled water.

Equipment. (Glassware should be acid cleaned.)

1. Photometer set at wavelength 515.
2. Test tubes of 15 ml. capacity.
3. Sahli pipet 0.02 ml. One ml. and 10 ml. volumetric pipets. The Sahli pipets should be calibrated[4] or should have a guaranteed accuracy of ±1 per cent.

Procedure.

1. For each unknown measure 1 ml. of the benzidine solution, Reagent 1, into a test tube. Add 2 extra tubes, one for the standard and one for the reagent blank.
2. Add 0.02 ml. of the unknowns and the standard Hb to the benzidine reagent. Mix by swirling.
3. Add 1 ml. of peroxide to each tube. Mix each immediately.
4. When the color change is completed (approximately 20 minutes) add 10 ml. of diluent Reagent 4. Mix by upending. Allow to stand 10 minutes. Transfer solutions to cuvettes and read in the photometer.
5. The optical density (*D*) of the standard represents a concentration of 20 mg. per 100 ml. (more or less; par. 3 under Reagents). *D* of the reagent blank is 0 and its Hb concentration is 0. On ordinary graph paper a line connecting the two points, 20 and 0, will provide corresponding values in mg. of Hb for the *D* of the unknowns.

Comments

Preparation of the unknown should provide an appropriate dilution to assure a photometer reading of maximum accuracy, an optical density in the range 0.1 to 0.6. When the concentration of hemoglobin is very high it may be advantageous to read the pigment as cyanmethemoglobin.[4] To increase the accuracy of the latter procedure the volume of plasma or urine added to the cyan reagent may exceed the 0.02 ml. used for hemoglobinometry of whole blood.

When the concentration of hemoglobin in the unknown is very low the accuracy of measurement may be increased in three ways. When the procedure is varied in this fashion it must be restandardized.

1. Increase the concentration of benzidine in Reagent 1. A 3 or 4 per cent solution may be used.
2. Increase the volume of unknown. The amount of plasma may be increased to 0.05 ml. without causing turbidity of any consequence. As much as 1 ml. of dialyzed urine may be used. (Add correspondingly less diluent in step 4.)
3. Decrease the volume of diluent. By using 4 ml. instead of 10 ml. the final volume is brought to 6 ml. instead of 12 ml. and the final color will be twice as dense.

The salts in urine cause turbidity and interfere with color development in the benzidine reaction. This may be obviated by dialyzing the urine overnight

Table 3.—*Measurement of Hemoglobin in Urine*

The percentages are corrected for the changes in volume that occurred during dialysis.

Tube	Optical Density	% Recovery in Terms of Tube 2	% Recovery in Terms of Tube 4
1. Urine & Hb. Standard	0.1278	49.2	
2. Saline & Hb. Standard	0.2596	100	
3. Dialyzed 1 (10% vol. increase)	0.2007	85	99.2
4. Dialyzed 2 (6% vol. decrease)	0.2366	85.6	
5. Dialyzed urine	0		

against 0.9 per cent NaCl solution. For comparison an aliquot of the standard hemoglobin solution, Reagent 3, should also be dialyzed. Results of the color reaction in the dialyzed urine should be compared with that of the dialyzed standard. The volume of both solutions is measured before and after dialysis and appropriate corrections are made of the final results (table 3).

The reagent blank should be almost colorless. Its *D* compared with distilled water is usually about 0.02. Darkening of the blank rouses a suspicion that reagents or glassware have been contaminated by traces of hemoglobin or other hemochromogens.

Substances that may be present in plasma as experimental additives can modify the benzidine color reaction. Carbonate, acetate, lactate, and cyanide, for example, cause a nonspecific darkening. Sulfate and urate precipitate benzidine. Lead and other heavy metals cause peculiar shades of color to develop. The usual anticoagulants, citrate, oxalate, heparin and EDTA, do not interfere.

When the proposed method is used in a routine fashion by a practiced technician over 30 specimens can be done per hour with an accuracy and reproducibility that is better than ±5 per cent.

Summary

A modification of the benzidine method for measuring hemoglobin in plasma and urine is described. When a known amount of hemoglobin is added to plasma or urine more than 95 per cent can be recovered with this procedure.

Summario in Interlingua

Es describite un modification del methodo a benzidina pro le mesuration de hemoglobina in plasma e urina. Post que un cognoscite quantitate de hemoglobina es addite a plasma o urina, le methodo permitte le recovration de plus que 95 pro cento de illo.

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