Sensitivity of phenolphthalein and benzidine tests in field of Forensic sciences

Ashutosh Sharma
Scientific Assistant, Forensic Science laboratory, Madhuban, Karnal, Haryana.)
Email - Ashutoshsharma.kaushik@gmail.com

Abstract: *The study gives the information that different stains confuse us at crime scene because they look like blood stains. Therefore sensitivity of benzidine and phenophtaline was important to be checked and it was checked with various vegetables and fruits. This was performed so that a result could be concluded on both which is more reliable to be used at the crime scenes. Therefore after the tests it was concluded that benzidine is more sensitive test than the phenolphthalein. Because most vegetables products and some other compounds gives false positive test with benzidine. But phenolphthalein does not found positive except turmeric. We have found the sensitivity 0f benzidine up to 1200000 times dilution to blood. And the sensitivity of phenolphthalein is found up to 300000 times dilution to blood.*

Keywords: *sensitivity, benzidine, combination, blood, DNA.*

1. INTRODUCTION:

Forensic serology is the science that deals with the study of various stains and implementing this study in combination with law. During a crime scene when such stains are encountered various steps are taken that for the identification of blood they are:

(1) Visual examination,
(2) Presumptive testing,
(3) Conformation testing ,
(4) Special identification ,
(5) individualization testing .

Blood evidence analysis has a long history. In the field of forensic science, blood is one of the most crucial forms of evidence. Blood evidence can help identify perpetrators by matching bloodstains with a reference sample. In order to obtain an identity based on blood, forensic scientists need accurate and reliable blood analysis methods. Therefore presumptive test is used for screening. There are various technologies that have improved the reliability of blood analysis such as DNA. But to reach to that point of research presumptive tests are most important and above that we should know which presumptive test is more sensitive and will give better desired results. Therefore, purpose this study to find out sensitivity of presumptive tests specifically benzidine and phenolphthalein (kastle Mayer) tests for identification of blood like stains found on crime scene. These test are also performed to know the sensitivity of these test on blood for different dilution.

2. BENZIDINE ($C_{12}H_{12}N_2$):

Since its discovery in 1904 benzidine has enjoyed both extremes of popularity and credibility. Early workers found benzidine to be a sensitive and specific test for blood. In time benzidine was discovered to be nonspecific for blood but specific for peroxidases. Because of its lack of specificity, man workers have discouraged its use. In 1964, Culliford and Nickolls published an in-depth review of the benzidine test. They found that false positives could be obtained from blood contamination, chemical oxidants, catalysts, and vegetable peroxidases. It was their contention that with a few precautions these interferences could be eliminated or explained.

Because of Culliford and Nickolls' work, the use of benzidine was bolstered. In 1975, the Forensic Science Foundation reported in a study that 51% of 215 responding forensic laboratories used benzidine as at least one of their presumptive tests. The remaining 49% in the study were distributed among seven other color tests, indicating that benzidine enjoys a reasonably widespread use in forensic work. As a result of the sporadic popularity of benzidine and its dangers in use , other tests have been devised. Most, such as σ-tolidine has been reported to induce neoplasm. Leucomalachite green and phenolphthalein are not true peroxidase tests but indicate the presence of a "nonspecific" oxidizing system.

Although benzidine is considered a hazardous substance, most serologists consider it too valuable to abandon. Benzidine was suggested as a possible carcinogen as early as 1964, but it was not until 1974 that the

Occupational Safety and Health Administration banned its use and manufacture in the United States . This ban has made finding an alternative method crucial.

In 1974, Holland et al reported on the synthesis of 3,3', 5,5 '-tetramethylbenzidine (TMB) and suggested its possible use in the detection of blood. In their study, TMB was characterized (by melting point, elemental analysis, and infrared, ultraviolet, and neutron mass radiography), and its carcinogenic activity was investigated. All tumors found in rats given TMB either were benign tumors at the sight of injection or were tumors normally accompanying aging in that strain of rats. The next logical step in determining the forensic value of TMB would be to determine its sensitivity, specificity and ease of use.

3. PHENOLPHTHALEIN ($C_{20}H_{14}O_4$):

In 1960, Hunt et al.conducted a survey of the orthotolidine (OTL), leucomalachite green (LMG), phenolphthalein (PhTh) and 'Luminol' tests. They determined that phenolphthalein was sensitive for blood in the solution form at dilutions of 1:10,000,000. But they also reported negative results with this test when applied directly to small spots of blood. An evaluation of the tetramethylbenzidine (TMB) test was reported by Garner, et al. in 1976. They observed no significant differences between the benzidine and tetramethylbenzidine (TMB) in tests in either sensitivity or specificity. Higaki, et al. compared the phenolphtalein and benzidine tests as presumptive tests. Their results indicate that plant peroxidases contribute to false positive results in the benzidine test only.

More recently, Cox focused on the four presumptive tests for blood, using phenolphtalein (PhTh), tetramethylbenzidine (TMB), leucomalachite green (LMG), and orthotolidine (OTL). His findings indicate that the TMB and OTL are the most sensitive of the group, and also the most likely to produce interference color reactions with plant peroxidases. The LMG test is the least sensitive of the four examined. The PhTh and LMG are the most specific of the four examined.

Moreover, the PhTh test is the best single test, based on its sensitivity of 1:10,000 with stained filter paper and cotton cloth and on the failure of the reagent to react with plant peroxidases.

Principle for phenolphthalein and benzidine test:-
These test is based on chemical oxidation of a chromogenic substance i.e phenolphthalein / benzidine by an oxidising agent used i.e H_2O_2. This reaction is catalysed by group of haemoglobin.

This test is carried out by first applying the solution of chromogen to a sample followed by addition of oxidising agent.

4. MATERIALS AND METHODS REQUIRE FOR PHENOLPHTHALEIN TEST:

4.1 General Requirements:-
Beaker ,dropper,watch man filter paper,sample,test tube, conical flask,measuring cylinder,spirit lamp,oven etc.

4.2 Chemicals:-
Phenolphthalein , KOH, Zink dust ,Distilled water , Ethanol, H_2O_2 =3%

4.3 Reagent prepration :-

Stock solution prepration:-

(1) Took phenolphthalein=2gm, KOH=20gm, Distilled water=100ml in a round bottom flask and was heated on a heating mental.
(2) a glass condenser was fixed with this flask.
(3) Refluxed the above mixture with 20gm Zink Dust until the solution become colourless.
(4) Took stock solution of phenolphthalein 2ml + distilled water=10ml + ethanol=2ml.
(5) Prepare 3% H_2O_2 By mixing 5ml of 30% H_2O_2 into 45ml of distilled water.

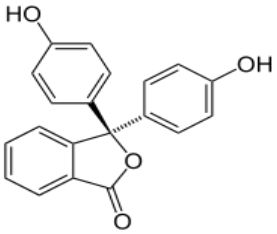
4.4 Procedure :-

(1) 2-3 drop of working solution of phenolphthalein were added to the stain either blood or other vegetarian product.

(2) After waiting to ensure that no colour develops at this stage then 1-2 drops of 3% H_2O_2 solution was added

(3) Note down the colour if pink colour is present in the sample it show that it may be blood.

4.5 Chemical structure of phenolphthalein:-

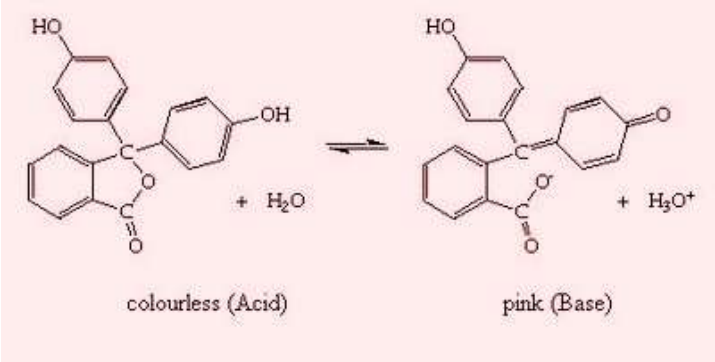


4.6 Reaction of the phenolphthalein with H_2O_2In the blood sample:-

Heme iron + phenolphthalein (clear) + H_2O_2 → oxidized phenolphthalein (pink) + H_2O + Heme iron

(In above reaction Heme iron is a catalyst and is unchanged)

4.7 Chemical reaction:-



Phenolphthalein (reduced) — add 3% H_2O_2 with Hb As Catalyst + Zn — phenolphthalein (oxidized)

5. MATERIALS AND METHODS REQUIRE FOR BENZIDINE TEST:

5.1 General Requirements:-

Beaker ,dropper, watman filter paper, sample, test tube, conical flask, measuring cylinder, spirit lamp, oven etc.

5.2 Chemicals:-

Benzidine , H_2O_2 ,Glacial acetic acid, H_2O_2 =3%,distilled water.

5.3 Reagent prepration:-

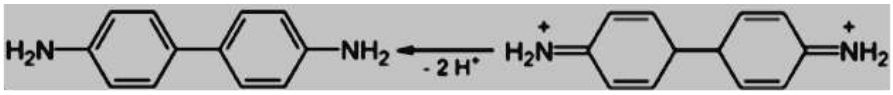
(1) Benzidine 7.5gm was mixed into 65ml of glacial acetic acid then diluted it with 285ml of distilled water.
(2) 3% H_2O_2 (5ml of 30% H_2O_2 Dissolve in 50ml of distilled water makes the reagent 3%)

5.4 Procedure :-

(1) 1-2 drop of benzidine solution + the given sample collected from crime scene or any other location.
(2) After waiting to ensure that no colour developed at that stage, 1-2 drops of 3% H_2O_2 solution was added
(3) Noted down the observation if blue colour is formed on the spot gives us information that it may be blood.

5.5 Chemical reaction:-

Hb + 3%H_2O_2



Benzidine reduced — benzidine oxidized

(blue colour formation)

All the above tests were performed with various vegetables , fruits and other items so that we could know which all other items also give positive tests for bezidine and phenolphthalein reagent

The table shown below gives us information about those vegetables,fruit or other items which gives the positive test with bezidine and phenolphthalein reagent.(here B indicate the benzidine and P indicate the phenolphthalein)

S.NO	Comman Name	Botenical Name	Family	PRESUMPTIVE TESTS			
				Original or fresh sample		After heating at 100C in oven for 20 minutes	
				B	P	B	P
1.	Turmeric (haldi)	Curcuma longa	Zingiberaceae	-	+	-	+
2.	Ginger (adrak)	Zingiber officinale	Zingiberaceae	+	-	+	-
3.	Garlic (lahsun)	Allium sativum	Liliaceae	+++	-	+++	-
4.	Onion (pyaz)	Allium cepa	Liliaceae	+	-	-	-
5.	Mint (pudina)	Mentha arvensis	Labitae	-	-	-	-
6.	Tusi	Ocimum sanctum	lamiaceae	-	-	-	-
7.	Tomato	Lycopersicon esculentum	Solanaceae	+	-	-	-
8.	Potato (aalu)	Solanum tuberosum	Solanaceae	+	-	-	-
9.	Brinjal (baingan)	Solanum melongena	Solanaceae	+	-	-	-
10.	Red pepper (lal mirch)	Capsicum annum	Solanaceae	-	-	-	-
11.	Green pepper (hari mirch)	Capsicum frutescens	Solanaceae	+	-	-	-
12.	Radish (muli)	Raphanus satvus	Cruciferae	+	-	-	-
13.	Cabbage (band gobhi)	Brassica aleraceae	Cruciferae	+	-	-	-
14.	Cauli flower (phul gobhi)	Brassica oleracea Var. botrytis	Cruciferae	+	-	-	-
15.	Lemon (nimbu)	Citrus limon	Rutaceae	+	-	-	-
16.	Orange (santra)	Citrus reticulata	Rutaceae	+	-	-	-
17.	Beet root (chukandar)	Beta vulgaris	Chenopodiaceae	+	-	-	-
18.	Spinach (palak)	Spinacia aleraceae	Chenopodiaceae	+	-	+	-
19.	Dhania	Coriandrum sativum	Umbelliferae	+++	-	-	-
20.	Heeng	Ferula asafoetida	Umbelliferae	+	-	-	-
21.	Carrot (gajar)	Daucus carota	Umbelliferae	+	-	-	-
22.	Fenugreek (methi)	Trigonella foenum-graceum	Papilionaceae	+	-	-	-
23.	Garden pea (matar)	Pisum sativum	Papilionaceae	+++	-	+	-
24.	Cucumber (khira)	Cucumis sativus	Cucurbitaceae	+	-	+	-
25.	Sitaphal (kadu)	Cucurbita maxima	Cucurbitaceae	+	-	+	-
26.	Gehoon (flour)	Triticum aestivum	Poaceae	+	-	+	-
27.	Dhub plant (bermuda grass)	Cynodon dactylon	Poaceae	+	-	-	-
28.	Sugar cane	Saccharum officinarum	gramineae	+	-	-	-

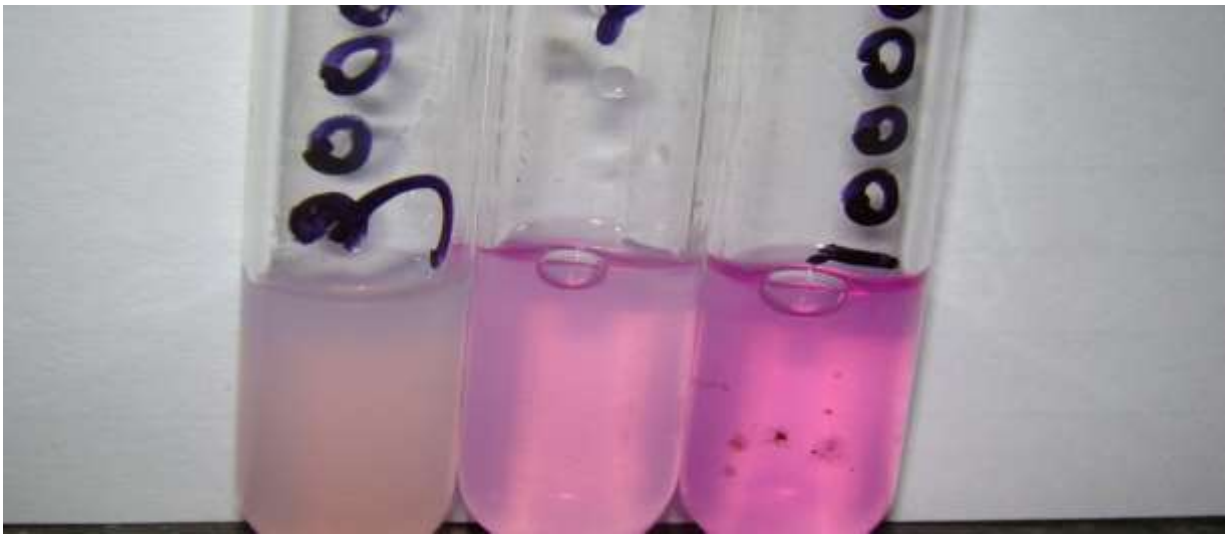
29.	Apple (save)	Pyrus malus	Rosaceae	+	-	-	-
30.	Grapes (angur)	Vitis vinifera	Vitaceae	-	-	-	-
31.	Banana (kela)	Musa paradisiace	Musaceae	+	-	-	-
32.	Pine apple (ananas)	Ananas comosus	Bromelia	+	-	-	-
33.	Guava (amrud)	Psidium guajava	Myrtaceae	+	-	-	-
34.	Neem leaves	Azadirachta indica	Meliaceae	-	-	-	-
35.	Peepul tree milk	Ficus religiosa	Moraceae	+	-	-	-
36.	Congress grass	Parthenium hysterophorus	Asteraceae	+	-	-	-
37.	Rape seed oil (sarson oil)	Brassica campestris	Brassicaceae	-	-	-	-
38.	Til oil	Sesanrum indicum L.	Pedaliaceae	-	-	-	-
39.	Panjiri ka patta	Anisochilus camosus	Lamiaceae	-	-	-	-
40.	Katha	Acacia catechu	Fabaceae	-	-	-	-
41.	Billi ka ghas	Oxalis dehradunensis	Oxalidaceae	+	-	-	-

Some chemical also gives benzidine and phenolphthalein reaction activities:-

S.No.	Salts	Benzidine	phenolphthalein
1.	Nacl	-	-
2.	Shop	-	-
3.	Glucose	-	-
4.	Sodium peroxide	-	-
5.	Lead acetate	-	-
6.	Ferrous sulphate	+	-
7.	Shikhar tombaco	-	-
8.	Rust	+	-
9.	Detergent(swaraj)	-	-
10.	Cold drink(dew)	-	-

Picture show sensitivity of benzidine In dilution of 100000%,500000%,100000%.

Picture show sensitivity of the benzidine on 1200000% dilution Of blood



Sensitivity of phenolphthalein on diluted blood of 100000%, 200000%, 300000%.

This Table is drawn for the sensitivity of benzidine and phenolphthalein on blood:- (these sensitive test done on the watch man filter paper up to 100000 time dilution of blood and more than these dilution is done in the test tube.)

Blood dilution	Benzidine reactivity (blue colour formation)	Phenolphthalein reactivity (pink colour formation)
Pure blood	+++	+++
5%	+++	+++
10%	+++	+++
50%	+++	+++
100%	+++	+++
500%	+++	+++
1000%	+++	+++
5000%	+++	+++
10000%	+++	+++
50000%	+++	++
100000%	+++	++
200000%	++	+
300000%	++	+
400000%	++	±
500000%	++	_
600000%	++	_
1000000%	+	_
1100000%	+	_
1200000%	+	_
1300000%	±	_
1400000%	_	_

Here shown dilution the +++ indicate the most reactive with blood (very deep colour formation) ,++ indicate the medium colour (no more light no more dark), + shows the very light colour formation.

6. DISCUSSION:

Our study gives the information that different stains confuse us at crime scene because they look like blood stains. Our study reveals that the benzidine is more sensitive test than the phenolphthalein. Because most vegetables products and some other compounds gives false positive test with benzidine . But phenolphthalein does not found positive except turmeric. We have found the sensitivity 0f benzidine up to 1200000 times dilution to blood. And the sensitivity of phenolphthalein is found up to 300000 times dilution to blood. But previous study reveal the sensitivity of phenolphthalein test with blood dilution of 100000 times (Delcarde and Benoit -1908 , Niconesco -1934). Some other

have found sensitivity of this test till 1×10^7time (Gettler and kaye -1943). Glaister (1926) noted that saline extracts of 1 year old stains reacted at 1 :212,000 dilutions but that a 1:800000 dilution of a water extract of the stain gave a positive result. And the researcher found the sensitivity of benzidine up to 100^5 to 300000 to 500000 (James stuard H. -1999).

7. CONCLUSION:

On the basis of our result , the phenolphthalein is found more suitable for routine case work for blood identification due to negligible sensitivity towards peroxidase enzyme of vegetables. And more safe than benzibine. Because bezidine gives false positive result. Benzidine is more sensitive than phenolphthalein.

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