

A STUDY OF THE SENSITIVITY, STABILITY AND SPECIFICITY OF PHENOLPHTHALEIN AS AN INDICATOR TEST FOR BLOOD

R. S. HIGAKI¹ and W. M. S. PHILIP¹

ABSTRACT

It has been known for many years that blood shows considerable peroxidase-like enzymatic activity and therefore in the presence of hydrogen peroxide will give highly coloured products with certain substrates and, in particular, with amines such as benzidine. The carcinogenic nature of benzidine, however, dictates a search for a satisfactory alternative substrate such as phenolphthalein which is believed to be non-carcinogenic. This study, designed to ascertain the relative merits of using benzidine and phenolphthalein as reagents in indicator tests for blood, centers primarily on the sensitivity, stability and specificity of the two substrates. Despite the fact that the three-stage phenolphthalein presumptive test for blood appears to be somewhat less sensitive than the conventional benzidine test, results indicate that it has several advantages in terms of specificity and stability. Results also indicate that the enzyme peroxidase, which is widely distributed in plants, does not contribute to false positive results in the three-stage phenolphthalein indicator test for bloodstains.

RESUME

On sait depuis longtemps que le sang donne lieu à des réactions enzymatiques importantes, du type peroxydase, et que, en présence de peroxyde d'hydrogène, il produit des substances vivement colorées avec certains substrats, en particulier avec des amines, telle la benzidine. Cependant la nature carcinogène de la benzidine exige que l'on recherche un autre substrat satisfaisant, telle la phénolphthaleïne, qui, l'on croit, n'est pas carcinogène. La présente étude se propose d'explorer les qualités relatives de la benzidine et de la phénolphthaleïne comme réactifs dans les tests indicateurs du sang. et porte surtout sur la sensibilité, la stabilité, et les caractères spécifiques de ces deux substrats. En dépit du fait que le test sanguin à la phénolphthaleïne en trois étapes semble moins sensible que le test ordinaire à la benzidine, les résultats indiquent qu'il comporte plusieurs avantages au point de vue de la stabilité et des caractères spécifiques. Les résultats démontrent également que l'enzyme peroxydase, qui existe à profusion dans les plantes, ne contribue pas à l'obtention de résultats positifs faux, lors du test indicateur à la phénolphthaleïne portant sur des taches de sang.

Introduction

Since the routine use of the benzidine test has been questioned due to its carcinogenic nature, a suitable replacement has been sought in our laboratory. The phenolphthalein test is by no means new, but because of its presumed safety and relative simplicity, it seemed to offer a suitable alternative to benzidine.

Safety and simplicity of use however, are not the only factors influencing the selection of one specific type of preliminary test. The sensitivity must be sufficient to detect the small and/or weak bloodstains encountered in casework. The stability of each reagent must be such that it has a suitable shelf-life and the test should possess a relatively high degree of specificity.

The purpose of the following experiments therefore, was to determine the sensitivity, stability and specificity of phenolphthalein.

PART I — Sensitivity

The purpose of part one was to assess the sensitivity of phenolphthalein relative

to benzidine.

Method: The phenolphthalin reagent was prepared using the method described in Lundquist(1). Serial dilutions of fresh human blood, neat to 0.2×10^{-6} were prepared and tested directly in solution form and in stain form. The direct test in stain form consisted of application of reagents to a stained thread on filter paper. The stains had been air dried for twenty-four hours.

The tests consisted of a benzidine control and twelve methods of application of the phenolphthalein test, (table I). The independently varied factors were peroxide vs methanol and one vs two vs three stage tests. A one stage test consisted of a single application of combined reagents.

A two stage test consisted of separate applications of two reagents.

A three stage test consisted of separate applications of three reagents.

All results were read at five seconds

¹ Biology Section, Centre of Forensic Sciences, 25 Grosvenor St., Toronto, Ont.. M7A 2G8.

except those for benzidine which were read immediately.

Results and Discussion

Regardless of the means of application, phenolphthalein compared quite favourably with benzidine for detecting blood in solution (Fig. 1). The peroxide based tests (2, 4 5 8 10, 11) were sensitive to dilutions of 10^{-4} to 10^{-5} approximately equivalent to benzidine, while tests using sodium perborate (3, 6, 7, 9,12,13) all surpassed the sensitivity of benzidine.

For detecting blood in stain form, the peroxide based tests had a maximum sensitivity of 10^{-3} (4, 5, 10, 11) (Fig. 2). The use of perborate did not measurably increase this sensitivity when directly testing dried stains. Benzidine was one hundred times more sensitive with an end point of 10^{-5} .

The use of ethanol or methanol with phenolphthalin increased the sensitivity by a factor of ten compared with the two stage method. Combining all reagents to form a one stage test had little effect on the results with the exception of the alcohol-perborate tests (12,13). In these cases, it was found that sensitivity was increased five times for blood in solution. However, under casework conditions, one infrequently deals with blood in solution form and to convert all stains into solution before screening is impractical. Only the methanol-perborate one stage test (13) was able to detect a 10^{-5} dried stain, a ten fold increase over other tests.

Considering sensitivity as the only criterion, it would appear that a one stage test consisting of a solution of equal quantities of methanol, phenolphthalin and sodium perborate would be the method of choice, followed by any of the three stage applications utilizing alcohol, phenolphthalin and either peroxide or perborate.

PART II — Stability

The purpose of part two was to determine the working life of the phenolphthalein reagents over a period of approximately six months under varying conditions of temperature, exposure to light and reagent mixtures.

Method: To approximate the casework conditions of stains of indeterminant age, stains of diluted human blood were prepared and allowed to age in parallel with test reagents. The reagents (Table 2) consisted of ethanol,

methanol, phenolphthalin, hydrogen peroxide and sodium perborate. Various storage conditions included room and refrigerator temperatures, amber and clear glass containers, separate reagent containers and reagents mixed together in a one stage test. The bloodstains were tested by the direct method with these reagents each week. A freshly prepared benzidine control was included with each series of weekly tests.

Results and Discussion

From the results (Fig. 3), one can see a rapid drop in the activity of all perborate based tests within the first three weeks of storage (I, J). The one stage peroxide test showed a similar drop after five weeks (H). Consequently, these two approaches were deemed insufficiently stable to be used in routine work and further tests were discontinued.

When stored separately, phenolphthalin and peroxide were found to be stable under varying conditions of temperature and exposure to light and gave good results when used with either ethanol or methanol for a period of at least 22 weeks (B-G).

The activity of the freshly prepared benzidine control dropped from 10^{-5} to 10^{-4} indicating a slight loss in activity of the stains with time. Phenolphthalein exhibited a similar drop but to a lesser degree. This decrease was expressed in the loss of borderline or weak positive reactions not shown in the graph.

From the point of view of stability, it appeared that a three stage peroxide based test with the reagents stored separately, was best and that all perborate and one stage tests were unsuitable.

PART III — Specificity

The purpose of this experiment was to determine the specificity of the phenolphthalein reaction relative to benzidine with respect to substances that would be expected to possess peroxidase or oxidative activity and/or have been found to produce some type of reaction with benzidine.

Method: Fourteen types of vegetable material, milk, household cleaners and various types of paper were tested. These substances were each directly tested in fresh form, as 1 day stains, and 4 day stains and as extracts of

9 week old stains. The tests applied were benzidine, 2 stage and 3 stage phenolphthalein.

Results and Discussion

Benzidine gave positive results with most of the vegetable material (Fig. 4). The three stage phenolphthalein test however produced only weak reactions with just four of the substances tested in fresh form. Of more significance was the fact that in the stain form, no false positives were obtained with phenolphthalein. This may be due to a higher degree of specificity, a lower level of sensitivity or a combination of the two.

From the sensitivity data, the three stage sodium perborate based tests were about ten times more sensitive to blood in solution than was benzidine. Therefore one would expect a positive phenolphthalein result in the extract test of those substances which gave positives with benzidine if the increased specificity was due to sensitivity. The lack of such evidence indicates that phenolphthalein is truly more specific for blood than is benzidine. One reason for this may be that the strongly alkaline phenolphthalin reagent serves to reduce or eliminate the interference from the plant peroxidases known to prefer a more acid substrate(2).

Only two of the materials tested, manila paper and tincture of iodine gave positive results before

the addition of peroxide. In the case of manila paper, a dark red color was obtained which suggests phenolphthalein should not be used when suspected stains adhere to this type of background. Tincture of iodine produced a weaker positive before the addition of peroxide. In both instances with the use of the three stage test, these results were clearly not false positives. It becomes obvious however, that a one stage test is not desirable because of reduced specificity.

Conclusions

From the results of this three part study of phenolphthalein as a presumptive test for blood, it can be concluded that:

- 1) For the direct testing of dried stains the three stage test is less sensitive than benzidine, but nevertheless sufficiently sensitive for general screening purposes where the detection of blood beyond dilutions of 10^{-3} is not required. In instances where greater sensitivity is required, the aqueous extract approach can render the test sensitive to a dilution of 10^{-5} (equal to the sensitivity of benzidine).
- 2) The three stage test using peroxide and either methanol or ethanol displayed maximum stability (ie. at least as stable as benzidine).
- 3) The three stage test using ethanol and peroxide was found to be significantly more specific for blood than was benzidine.

PART I

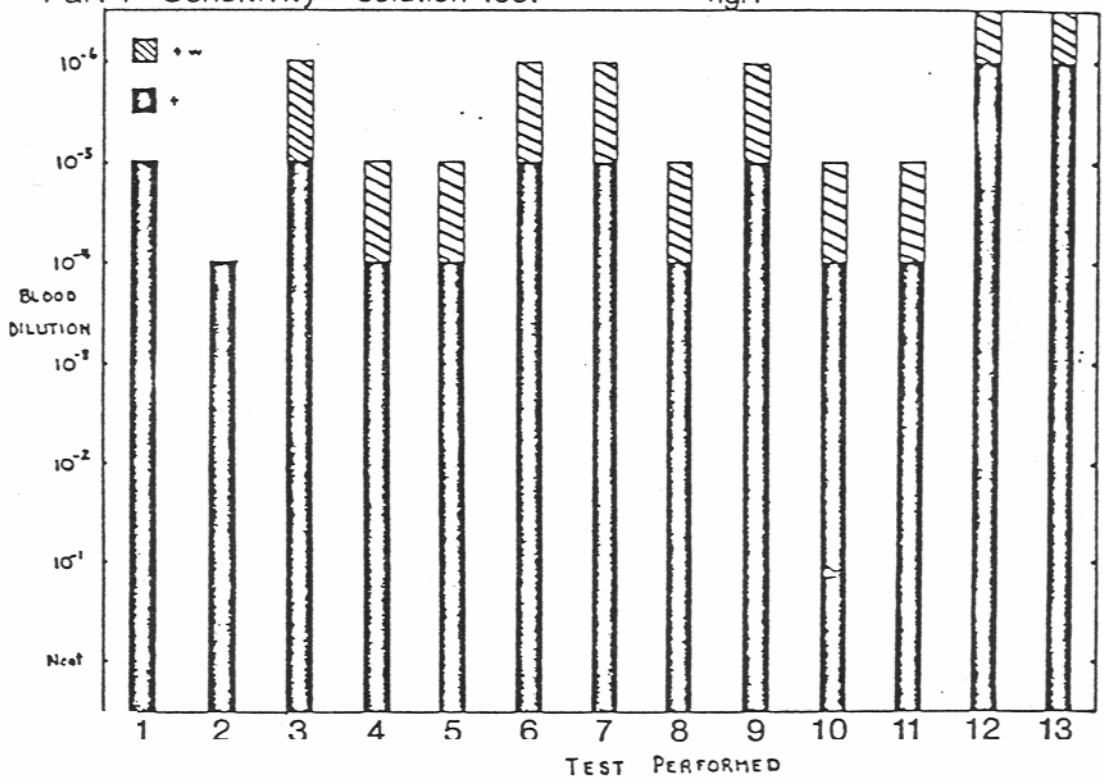
TABLE 1
SENSITIVITY

1) Two Stage	— Benzidine-Peroxide
2) Two stage	— Phenolphthalin-Peroxide (6%)
3) Two stage	— Phenolphthalin-Perborate (1.4%)
4) Three stage	— Ethanol-Phenolphthalin-Peroxide
5) Three stage	— Methanol-Phenolphthalin-Peroxide
6) Three Stage	— Ethanol-phenolphthalin-Perborate
7) Three stage	— Methanol-Phenolphthalin-Perborate
8) One stage	— Phenolphthalin + Peroxide Mixed Together
9) One stage	— Phenolphthalin + Perborate Mixed Together
10) One stage	— Ethanol + Phenolphthalin + Peroxide Mixed Together
11) One stage	— Methanol + Phenolphthalin + Peroxide. Mixed Together
12) One stage	— Ethanol + Phenolphthalin + Perborate Mixed Together
13) One stage	— Methanol + Phenolphthalin + Perborate Mixed Together

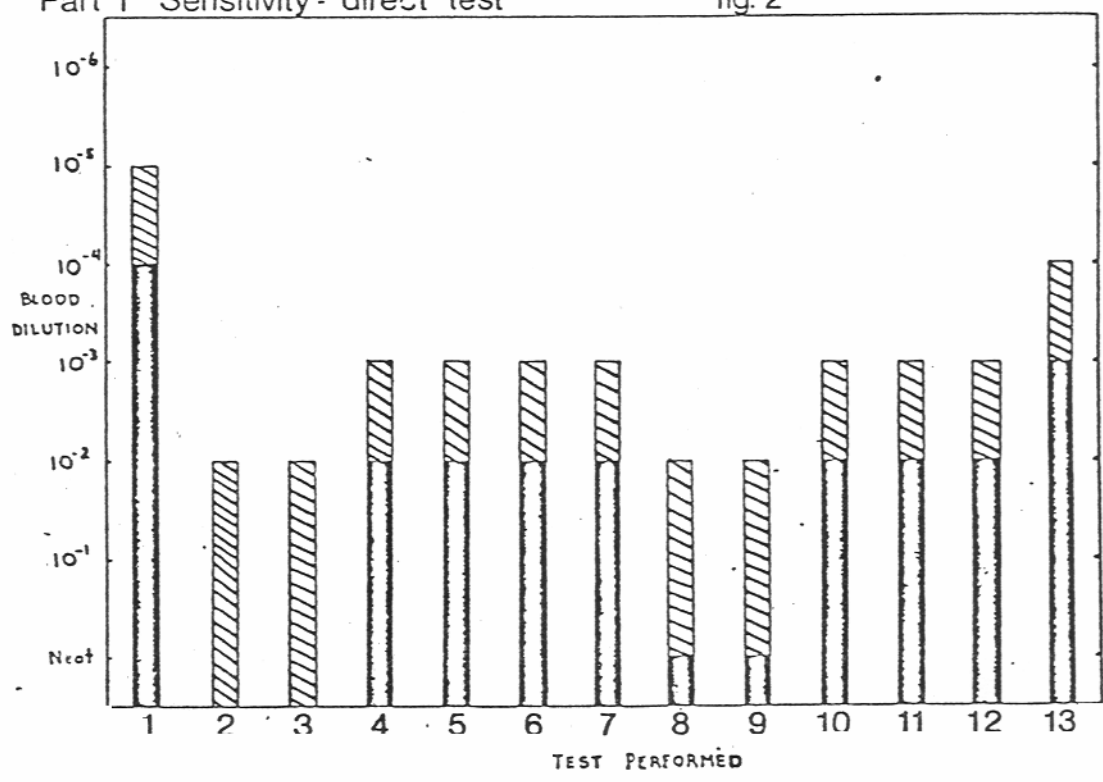
TABLE 2
STABILITY

- A) Control Benzidine — Fresh solutions prepared weekly
- B) Control Phenolphthalein — Fresh solutions from laboratory stock
 - Two stage peroxide
 - Three stage peroxide using:
 - i) ethanol
 - ii) methanol
- C) Aging Phenolphthalin and Peroxide —
 - Clear glass bottles, room temperature
 - Two stage, Three stage Ethanol, Methanol
- D) Aging Phenolphthalin and Peroxide —
 - Amber glass bottles, room temperature
 - Two stage, Three stage Ethanol, Methanol
- E) Fresh Phenolphthalin, Aging Peroxide —
 - Amber glass bottles, room temperature
 - Two stage, Three stage Ethanol, Methanol
- F) Aging Phenolphthalin, Fresh Peroxide —
 - Amber glass bottles, room temperature
 - Two stage, Three stage Ethanol, Methanol
- G) Aging Phenolphthalin and Peroxide —
 - Amber glass bottles, 4 C (refrigerator)
 - Two stage, Three stage Ethanol, Methanol
- H) One Stage Tests — Amber glass bottles, room temperature
 - i) Phenolphthalin + Peroxide
 - ii) Ethanol + Phenolphthalin + Peroxide
 - iii) Methanol + Phenolphthalin + Peroxide
- I) Aging Phenolphthalin and Perborate —
 - Amber glass bottles, room temperature
 - Two stage, Three stage Ethanol, Methanol
- I) One Stage Tests — Amber glass bottles, room temperature
 - i) Phenolphthalin + Perborate
 - ii) Ethanol + Phenolphthalin + Perborate
 - iii) Methanol + Phenolphthalin + Perborate

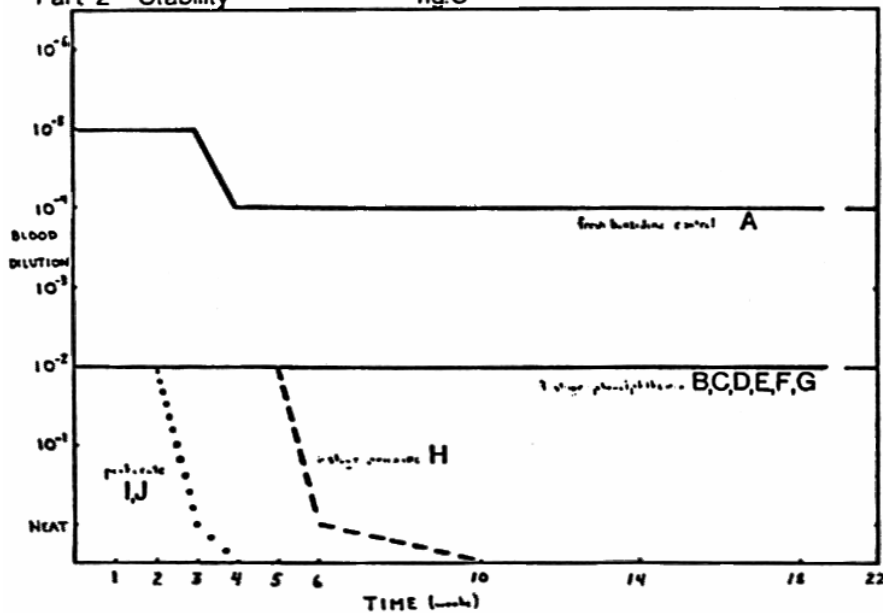
Part 1 Sensitivity - solution test. fig.1



Part 1 Sensitivity - direct test fig.2



Part 2 Stability fig.3



Part 3 Specificity fig.4

	2-stage BENZIDINE				3-stage PHENOLPHTHALEIN					
	benzidine-H ₂ O ₂				eth. phth-H ₂ O ₂			NaBO ₃		
	DIRECT	1 DAY STAINS	4 DAY STAINS	EXTRACT	DIRECT	SOLUTION	1 DAY STAINS	4 DAY STAINS	EXTRACT	EXTRACT
CHERRY	-	-	-	-	-	-	-	-	-	-
TOMATO	-	-	-	-	-	-	-	-	-	-
CABBAGE	-	-	-	-	-	-	-	-	-	-
LETTUCE	-	-	-	-	-	-	-	-	-	-
CUCUMBER	-	-	-	-	-	-	-	-	-	-
GREEN BEAN	-	-	-	-	-	-	-	-	-	-
POTATO	-	-	-	-	-	-	-	-	-	-
ONION	-	-	-	-	-	-	-	-	-	-
RADISH	-	-	-	-	-	-	-	-	-	-
CARROT	-	-	-	-	-	-	-	-	-	-
GARLIC	-	-	-	-	-	-	-	-	-	-
ORANGE JUICE	-	-	-	-	-	-	-	-	-	-
MILK	-	-	-	-	-	-	-	-	-	-
HORSE RADISH	-	-	-	-	-	-	-	-	-	-
PREPARED MUSTARD	-	-	-	-	-	-	-	-	-	-
POTASSIUM PERMANGANATE	-	-	-	-	-	-	-	-	-	-
SOK FROM SEWER	-	-	-	-	-	-	-	-	-	-

REFERENCES

- (1) Lundquist, F. Methods of Forensic Sciences. Vol. 1, publ. by Interscience Publishers 1962, p. 254-255.
- (2)- Cuffliford, B.J. The Examination and Type of Bloodstains in the Crime Laboratory, publ. by U.S. Dept. of Justice, Dec. 1971, p. 44-51.

ACKNOWLEDGEMENT

The authors wish to thank Mr. D. M. Lucas, Director and Mr. S. E. Brown, Head of Biology Section, Centre of Forensic Sciences, Toronto, for their valuable advice in the preparation of this paper.