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New reagents for detection of faecal occult blood

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Abstract

Imipramine hydrochloride (IPH) and desipramine hydrochloride (DPH), two widely used antidepressant drugs, are proposed as new reagents for detection of faecal occult blood. The usefulness of IPH and DPH in occult blood detection has been examined and compared with benzidine and stanoccult methods. The results show that the proposed reagents are selective and sensitive and gives reproducible results. The proposed methodology is much less subject to vegetable peroxidase, iron and vitamin C interference and can be performed on patients who are on a normal diet.

Keywords: Imipramine hydrochloride; Desipramine hydrochloride; Faecal occult blood; Detection; Chemical method

1. Introduction

Tests for the presence of occult blood in faeces form an important part of the investigation of anaemia [1] and gastrointestinal disease [2], and for the early detection of colorectal cancer [3–7] which is the second leading cause of cancer deaths in Western society [4,7]. The usefulness of this approach, however, has been hampered by the unreliability of many screening methods [8], because, the incidence of false-positive [8] and false-negative [9] results in subjects who are on an unrestricted diet, or on vitamin C may be very high.

Interference from vegetable peroxidase, enzymes present in many types of vegetables and fruit, is also a problem. These enzymes demonstrate some resistance to heat denaturation and may not be inactivated by cooking. Dietary restriction of both haemoglobin and vegetable peroxidase containing substances is therefore essential for valid screening for occult blood. One approach [10] to reducing intereferences at the assay stage has been to boil faecal samples thoroughly before analysis, thus inactivating the vegetable enzymes while leaving the pseudoperoxidase activity of haemoglobin largely intact. Though this procedure is inconvenient when large numbers of samples are assayed, it has a distinct advantage — the false negative results due to ascorbic acid is almost eliminated [11].

The methods available for the detection of faecal occult blood fall in four categories; viz. radio-

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analytical, physical, immunochemical and chemical methods. Determination of radioactivity in faeces after intravenous injection of ^{51}Cr-labeled red blood cells is specific for quantitative analysis, but too complicated for routine use. Physical methods, such as microscopical examination of faeces for red blood cells and haematin crystals, or spectroscopic identification of haemoglobin and its derivatives, have been suggested. However, these methods have remained academic curiosities. The immunochemical methods are specific, selective and reproducible. However, the process is time consuming.

Among the numerous methods reported in the literature for the detection of faecal occult blood, attention has chiefly been concentrated on chemical methods which are based on the pseudoperoxidase activity of hematin portion of the haemoglobin molecule. The catalytic cleavage of hydrogen peroxide by haemoglobin, and certain of its dervatives, can be coupled with an indicator reaction in which a chromogenic oxygen acceptor is oxidized to its coloured form.

The chromogens proposed for the detection of faecal occult blood fall into two types: carcinogens and non-carcinogens. Historically, benzidine, a hazardous carcinogen [12] has been used as a reagent for almost a century. The sensitivity and specificity of the reagent are well illustrated by the fact that, inspite of extreme hazards associated with exposure to this compound, there has been a marked reluctance to abandon its use, for no suitable substitute has hitherto been found. Incidentally, the carcinogenic chromogens, as also the chromogens which are thought to be carcinogens because of their chemical structure, are sensitive (Table 1). While, the non-carcinogenic chromogens proposed are unsatisfactory for one reason or another.

The deficiencies of the existing methods encouraged the authors to develop safer substitutes which are of paramount importance. In the present communication, we have investigated *N*-substituted dibenzazepines, viz. imipramine hydrochloride (IPH) and desipramine hydrochloride (DPH), as a new class of chemical reagents for the detection of faecal occult blood (Table 2). In the presence of acetic acid and hydrogen peroxide both these chromogens give rise to a green-blue colour with blood; the development of this colour has been explored under clinical laboratory bench top conditions using human faecal samples to which blood had been added.

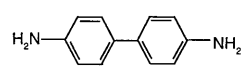
2. Materials and methods

Stools were collected for two days and nights from three healthy persons who were kept on a diet free of meat, fish and peroxidase containing vegetables for 72 h prior to collection. No drugs were given. The stools so collected were tested with benzidine reagent which gave negative results for the blood. 1–7 ml of human blood obtained from the blood bank was added to seven different beakers containing 100 g of faeces. The mixture was homogenized. The sensitivity of the proposed reagents were tested with reference to benzidine method.

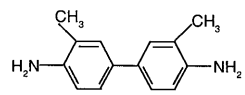
Table 1
Structure of the chromogens for the detection of blood in urine

HAZARDOUS CARCINOGENS

1) BENZIDINE

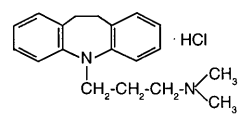


2) o-TOLIDINE



PROPOSED REAGENTS

1) IMIPRAMINE HYDROCHLORIDE



2) DESIPRAMINE HYDROCHLORIDE

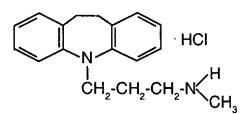


Table 2
Detection of faecal occult blood

Volume of blood[a]/100 g feces[b] ml	Method		
	IPH	DPH	Benzidine
1	Tr[c]	Tr	Tr
2	Tr	Tr	+
3	+	+	2+
4	2+	2+	3+
5	3+	3+	4+
6	4+	4+	4+
7	4+	4+	4+

[a] Human blood obtained from blood bank.
[b] Samples obtained from healthy persons who were kept on diet free of meat, fish, peroxidase containing vegetables and drugs. The mixture of human blood and the faeces were homogenized.
[c] Tr, trace.

2.1. Reagents

Two gram of IPH or DPH and 0.4 g of EDTA dissolved in 100ml of glass distilled water and stored in an amber bottle. Hydrogen peroxide (10% w/v) and glacial acetic acid were procured from Glaxo India Ltd.

2.2. Procedure

In a test tube suspend about 2 g of faeces in 5 ml of water. Place the test tube in boiling water in a water bath for 2–3 min in order to destroy peroxidase activity and ascorbic acid and cool to room temperature. Place 0.2 ml of reagent solution containing EDTA and IPH or DPH, 0.2 ml of glacial acetic acid, 0.1 ml of boiled faeces suspension and 0.05 ml of 10% volume hydrogen peroxide on a porcelein spot plate and mix well. Keep it aside for about 2 min at room temperature. The appearance of green-blue to blue colour is to be regarded as a positive result indicating the presence of blood in faeces.

2.3. Investigations

The following investigations were carried out using (1) benzidine test, (2) Stannoccult; and (3) the proposed dibenzazepine test.

2.3.1. Test on iron

In vitro, occult blood tests were made on the solutions of the iron compounds in current therapeutic use.

1. 0.4% w/v of Ultiron TR capsules that contains ferrous sulphate (Sidmark laboratory, India).
2. 0.4% w/v of persolate tablet that contains ferrous sulphate (Glaxo, India).
3. 0.1% w/v ferrous sulphate containing a few drops of acetic acid. IPH and DPH do not give positive results.

Volunteers (same persons whose stools were taken for our experiments) on a meat and fish-free diet were given two iron tablets per day for three days. The stools were tested for occult blood. IPH or DPH method gave negative results, while benzidine and stanoccult gave false positive results.

In vivo ascorbic acid upto 2000 mg/day did not give false negative results with IPH or DPH but interfered with benzidine and stanoccult method. Boiling of faecal suspension is also not necessary at the aforesaid ascorbic acid dosage. For higher concentration of ascorbic acid (>3 g/day) boiling of the faecal sample is desirable.

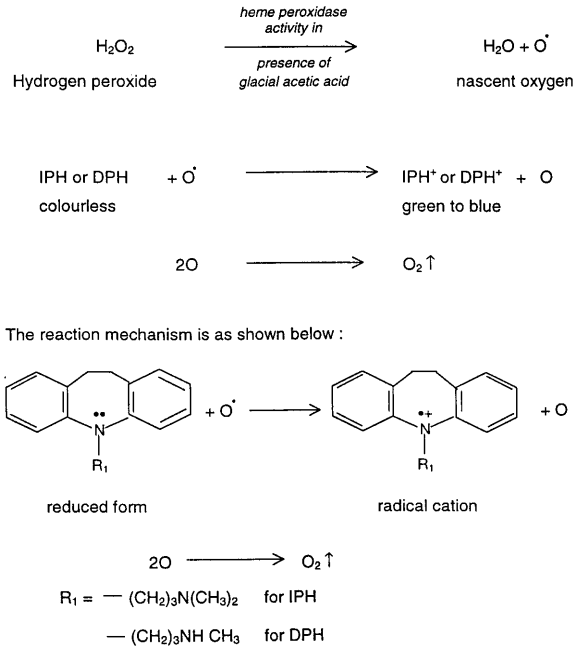
2.3.2. Comparison of benzidine, Stanoccult, IPH and DPH methods

One hundred and seventy eight samples for tests on occult blood in a leading hospital were studied with methods of benzidine, Stanoccult, IPH and DPH. Each sample was tested independently six times by the authors and two technicians in a hospital. The results are presented in Table 3.

3. Results and discussion

IPH and DPH belong to an important class of tricyclic antidepressant drugs having a central ring constituted of seven atoms. They are extensively used for the treatment of depressive disorders, and their efficacy in alleviating depression has been well established [13].

Pseudoperoxidase activity of heme in haemoglobin liberates nascent oxygen from hydrogen peroxide in acetic acid medium. The liberated oxygen oxidizes IPH or DPH as per the following reaction:



3.1. Effect of EDTA on metals

The above reaction falls under the category of chemical oxidation process, hence, we studied the effect of commonly used oxidizing agents, such as iron(III), chromium(VI), cerium(IV), vanadium(V) and potassium ferricyanide, on IPH or DPH. All the above oxidizing agents yield green/blue colour in lower pH region (pH < 4). Of the above reagents tested iron is important in the present investigations, because of its extensive therapeutic use. That interference due to iron leads to false-positive results, with most of the chromogens is an established fact. To eliminate the interference due to iron we used 0.4% (w/v) EDTA while preparing IPH or DPH reagent. In an earlier communication [14] sodium salt of *N*,*N*-di-(2 hydroxyethyl) glycine was reported to have been used in the reaction mixture, to obviate interference of iron. However, disodium salt of ethylenediamine tetraacetic acid (EDTA) has the following advantages: first of all, EDTA is a hexadenate ligand, thus forms stable chelate complexes with iron(II) and iron(III); and second, EDTA as a versatile complexing agent complexes with metal ions such as cobalt, copper, zinc, calcium, magnesium which are likely to be a part of a therapeutic dosage. The use of EDTA [15] and a few of its advantages have been reported by us earlier [11].

3.2. Effect of metabolites

Investigations were carried out on the faecal samples collected from patients who were on IPH or DPH drug and suffering from disease associated with faecal occult blood. Our purpose was to study the interference of metabolic products of IPH or DPH in detection of faecal occult blood by the proposed methods. It was difficult to arrive at a definite conclusion, as to whether the metabolites interfere during the selection process. Hence

Table 3
Detection of occult blood in pathological human faecal samples[a]

Suspected disease	No. of samples	Benzidine method			Stanoccult method			IPH or DPH		
		Neg[a]	Tr[b]	Positive[c]	Neg	Tr	Positive	Neg	Tr	Positive
Anaemia	62	34	18	10	36	16	10	28	14	10
Ulcer	32	18	12	2	20	10	2	21	9	02
Gastritis	42	28	10	4	28	10	4	30	8	04
Gastrointenstinal malignancy	26	08	04	14	08	05	13	08	05	13
Colorectal cancer	16	07	05	04	08	04	04	08	04	04

[a] Neg: negative — if no colour develops after 3 min.
[b] Tr: Trace — if feeble colour appears after 2 min.
[c] Positive: if, colour appears within 2 min.

in vitro studies were carried out to study the interference of metabolites of IPH or DPH.

IPH is metabolized primarily by two pathways: *N*-demethylation and hydroxylation [15]. Removal of one methyl group from the aliphatic side chain results in the formation of desipramine [DPH], an active metabolite also efficacious for treatment of depression, which has been proposed as a reagent in the present context. Hydroxylation of imipramine on the aromatic ring produces 2-hydroxyimipramine, the major hydroxylated metabolite. Removal of the second methyl group from the aliphatic side chain, oxide formation to imipramine *N*-oxide, and finally cleavage of the entire aliphatic side chain to make iminodibenzyl have also been reported in the literature. Thus, we tested for the interference of 2-hydroxy imipramine and iminodibenzyl, a final metabolite product. No interference was observed.

3.3. Effect of acid

Many experiments were carried out on the faeces samples homogenized with blood in the laboratory, and pathological samples collected from a hospital using different acid media, viz. sulphuric, hydrochloric, perchloric, phosphoric and acetic acid media. Higher percentage of false-positive results were observed in the case of sulphuric and hydrochloric acid media. Erratic behaviour of the development of colour in perchloric acid medium and poor reproducibility in the case of phosphoric acid medium were observed. In contrast, acetic acid medium gives better results in terms of reproducibility and stability of the colour. Thus, acetic acid medium has been selected for experiments.

3.4. Sensitivity and reproducibility

Our results on the experiments carried out on stool samples homogenized with human blood (obtained from blood bank) indicate that the intensity and the rate of development of colour depend on the amount of blood present in faeces. The sensitivity of IPH or DPH is 3 ml per 100 g of faeces, while the limit of detection is 1 ml per 100 g of faeces. The value of standard deviation of 4.5 obtained indicate that the method is reliable because of good reproducibility. A blank experiment (without blood or peroxidase like activity) was carried out with IPH or DPH reagent, hydrogen peroxide and glacial acetic acid. No colour was seen even after 24 h. The results are presented in Table 2.

We have also investigated in detail the other members of dibenzazepine class of compounds, namely, trimipramine maleate, clomipramine hydrochloride and opipramol. Trimipramine maleate (low solubility in water) give blue colour with blood in faeces. However, its sensitivity is poor (colour is seen with concentrations greater than 8 ml of blood per 100 g of faeces). The other two members viz. clomipramine hydrochloride and opipramol do not display any colour under the conditions investigated.

3.5. Studies on benzidine, Stanoccult, IPH and DPH methods

Examination of faecal samples for occult blood loss is so common in developing countries that screening procedures need to be simple and cost-effective, both for the patient and the laboratory. This is all the more important as many patients continue to take an unrestricted diet. Though benzidine, which was previously used, is sensitive, it has serious disadvantages of giving false positive results with iron, and false-negative results on faecal samples obtained from patients who are on vitamin C. Stanoccult is widely used in our country for visual qualitative test for occult blood in faeces. The identity of the chromogen is not revealed by the manufacturer. However, the literature indicates that *o*-toluidine, which is a hazardous carcinogen, probably used as the chromogen.

4. Conclusion

The proposed IPH and DPH are relatively inexpensive and have the required sensitivity as is manifested by our results. Though the proposed method has some minor procedural disadvantage

of boiling (only in case of patients who are on more than 3 g of vitamin C per day) it is simple to perform. However, the method offers advantages of relatively low sensitivity to endogeneous vegetable peroxidases; hence, the test can be performed on samples taken from patients who are on a normal diet, and the results are reproducible. The time tested IPH and DPH offers an unshakable guarantee that the reagents are non-carcinogenic and can replace benzidine and *o*-toluidine without any extra procedural difficulties as they also exhibit similar blue colour.

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