ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 501–507

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Imipramine hydrochloride and desipramine hydrochloride as new reagents for detection of microamounts of blood in urine

Akheel A. Syed *, Mohammed F. Silwadi, Bibi A. Khatoon

Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570 006, India

Received 30 August 2001; received in revised form 27 September 2001; accepted 5 October 2001

Abstract

Benzidine and *o*-tolidine, the hazardous carcinogens are still in use for the detection of blood in urine. Development of safer substitutes are of paramount importance. Unfortunately, the alternate available reagents lack specificity, sensitivity and reproducibility. Imipramine hydrochloride (IPH) and desipramine hydrochloride (DPH) are proposed as new reagents for the detection of blood in urine. Both the reagents impact to blood a green–blue to blue color, in the presence of acetic acid and hydrogen peroxide and the development of this color is explored under laboratory conditions. IPH or DPH compares favorably with benzidine and multistix methods. Both the reagents are relatively inexpensive, and have required sensitivity and stability. The proposed reagents offer advantages of relatively low sensitivity to endogenous vegetable peroxidases; hence, the test can be performed on samples taken from patients who are on a normal diet and the results are reproducible. Besides, the reagents, are non-carcinogenic and can replace benzidine and *o*-tolidine without any extra procedural difficulties as they also exhibit a similar blue color.

Keywords: Imipramine hydrochloride; Desipramine hydrochloride; Blood in urine; Detection; Chemical method

1. Introduction

Urine analysis has long been regarded as an indicator of a patient's state of health. The ease with which urine may be obtained and the patient's willingness to provide a urine specimen make urine analysis one of the most commonly performed screening examinations [1]. Examination of urine may be considered from two general stand points: (i) diagnosis and management of renal or urinary tract disease; and (ii) the detection of metabolic or systemic diseases not directly related to the kidney. The finding of blood amongst various constituents present in urine may indicate the presence of a pathological condition, of renal origin, within the urinary tract. Blood may be present in the urine either as red blood cells, or as hemoglobin. The former are found in cases of haematuria—a relatively common finding which has many potential underlying causes,

* Corresponding author. Fax: +91-821-518835/421263.
E-mail address: syedakheelahmed@hotmail.com (A.A. Syed).

0731-7085/02/$ - see front matter
PII: S0731-7085(01)00665-3

ranging from the physiological to life-threatening—while the latter is seen in the much less frequently occurring cases of haemoglobinuria.

The physiological disorders commonly associated with haematuria include pyelonephritis, kidney stones and lupus nephritis; on the otherhand, tumors of the genitourinary tract are a manifestation of underlying malignant disease [2]. Gross haematuria is also known to be the presenting sign in as many as 85% of patients with bladder cancer [3], and in as many as 40% of patients with renal cell carcinoma [4]. Any disorder associated with hemolysis of red blood cells, and the resultant release of haemoglobin, may lead to the appearance of haemoglobin in urine. Common causes of haemoglobinuria include haemolytic anemias, transfusion reactions, trauma to red blood cells by prosthetic cardiac valves, extensive burns, trauma to muscles and blood vessels, and severe infections.

Haematuria is a finding which requires careful clinical management. Hence, it is mandatory, in every case of haematuria, to localize the site of the bleed, as a protocol for the investigations and for the management for patients with physiological causes and life-threatening diseases [5]. For efficient management, a simple and cost-effective diagnosis test is of paramount importance, especially in developing countries where the high costs of hospital stay and expensive procedures are not affordable. The techniques that are now available for the diagnosis of disorders producing bleeding include, urine microscopy; urine cytology; urine-based marker tests; cytoscopy; ultrasonography; computed tomography (CT); excretion urography; volumetric analysis; magnetic resonace imaging (MRI); angiography; nuclear medicine; renal biopsy and chemical tests. Of the aforesaid techniques, the commercially available laboratory reagent strips have been demonstrated to be more promising, as they have the advantages of speed, convenience, cost-effective and of not being confined to the laboratory. Therefore, it is not surprising that chemical tests in the form of dipstick have become popular and widely used.

Dipstick measurement of occult blood as a replacement for microscopic examination has been evaluated by different research groups [6–10]. The causes of false-positive [11,12] and false-negative [10,11] results have also been reported in the literature. Experiments to eliminate the interference of vegetable or bacterial peroxidase by heat, or by addition of an inhibitor, were not entirely successful in Hemastix, Labstix reagent strip methods [13]. Furthermore, the sensitivity of Multistix and Chemstrip decreases with the age of the reagent strip [14].

All the chemical methods are based on pseudoperoxidase activity of hematin portion of haemoglobin molecule. The catalytic cleavage of hydrogen peroxide by haemoglobin, and certain of its derivatives, can be coupled with an indicator reaction in which a chromogenic oxygen acceptor is oxidized to its colored form.

The chromogens proposed for the detection of occult blood fall into two types, carcinogens and noncarcinogens. Historically, benzidine [15], a hazardous carcinogen [16] 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, because no suitable substitute has hitherto been found. The substitutes reported in the literature are displayed in Fig. 1. Incidentally, the carcinogenic chromogens, as also the chromogens which are thought to be carcinogens because of their chemical structure, are sensitive. For example, *o*-tolidine, an alternative benzidine class of reagent, has also been reported to be a hazardous carcinogen [16]; 3,5,3′,5′-tetramethyl benzidine [17] may be considered as unsatisfactory because of the possibility that it also exhibits significant carcinogenesis. The non-carcinogenic chromogens for the detection of blood in urine exhibit deficiencies when used in routine laboratory methods. N-substituted phenonthiazins, reported by us earlier [18] as reagents for the detection of micro amounts of blood in urine, produce a red color which is different from the blue color produced by reagents of the benzidine class. This is important, as routine tests in hospitals are generally carried out by novices. Thus, any color other than blue-green is difficult to adopt. The Kastle–Meyer

reagent (reduced phenolpthalein) is troublesome to prepare due to its caustic nature, and thus unsuitable for domiciliary practice [19]. The guaic test is expensive, insensitive, and not very reliable, and should be abandoned [19]. However, in a recent communication [20] it is reported that interference of plant peroxidases with guaiac-based fecal occult blood tests is avoidable. These deficiencies have stimulated the authors to seek alternative reagents which are of paramount importance. Recently, we have reported the investigations on a new class reagents, viz., N-substituted dibenzazepines viz., imipramine hydrochloride (IPH) and desipramine hydrochloride (DPH) as safe alternatives for the detection of fecal occult blood [21]. In this paper, we report the use of the same reagents to detect micro amounts of blood in urine. Both these chromogens produce, in the presence of acetic acid and hydrogen peroxide, a green–blue to blue color with blood; and the development of this color has been explored under clinical laboratory conditions, using human urine samples to which blood had been added.

2. Materials and methods

Urine samples were prepared by homogenizing a known volume of human blood (from a blood bank) in 100 ml of urine obtained from ostensibly healthy persons, who were kept on a diet free from meat, fish, and peroxidase containing vegetables and drugs for 72 h before the collection.

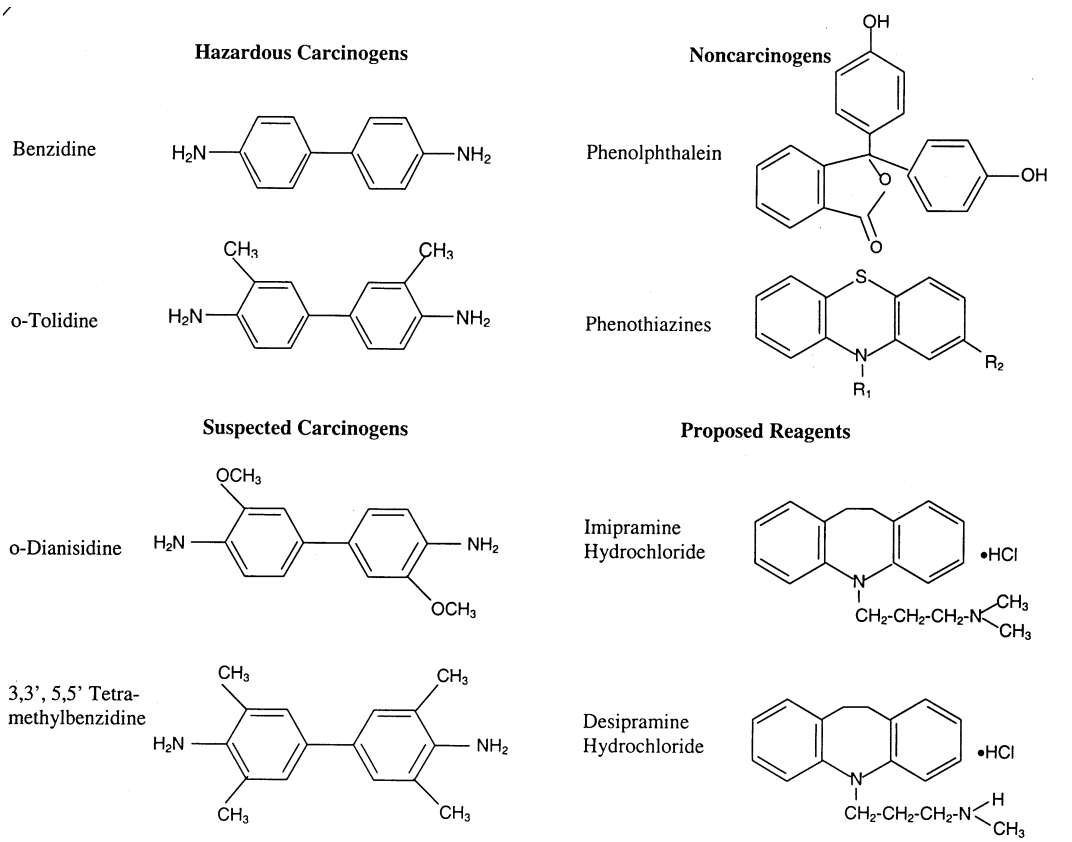

Fig. 1. Structures of the chromogens for the detection of blood in urine.

Table 1
Detection of hematuria

Volume of blood per 100 ml urine (μl)[a,b]	Benzidine	IPH	DPH
2	−	−	−
5	−	−	−
10	Tr	−	−
20	Tr	Tr	Tr
30	+	Tr	Tr
40	2+	+	+
50	3+	2+	2+
60	4+	3+	3+
70	4+	4+	4+

−, Negative; Tr, trace; lacks reproducibility and color development takes >3 min; +, indicates weakly positive and development of color; >2 and <3 min; 2+, positive; development of color >1 and <2 min; 3+, the color develops <1 min; 4+, indicates development of color within 30 s. 2+, 3+, 4+ can be considered definitely positive, while, Tr displays poor reproducibility and + lacks reliability of the results.

[a] Human blood obtained from blood bank.

[b] Samples obtained from healthy persons who were kept on diet free of vitamin C, meat, fish, peroxidase containing vegetables and drugs. The mixture of human blood and urine were homogenized.

2.1. Reagents

Hydrogen peroxide (10% w/v) and Glacial acetic acid.

Two g of IPH or DPH and 0.4 g of EDTA (to remove interfering metals by complexation) were dissolved in 100 ml of glass distilled water; the solution was stored in an amber bottle.

Oxidised IPH or DPH solution was prepared by treating 0.2 ml of the previously prepared IPH or DPH solution with 0.05 ml of approximately 1:1 nitric acid. The blue color obtained by the oxidation of IPH or DPH with nitric acid is very unstable, and hence, it should be used within the shortest possible duration of time.

2.2. Procedure

Place 0.2 ml of IPH or DPH reagent, 0.3 ml of glacial acetic acid, 0.1 ml of urine sample and 0.1 ml of hydrogen peroxide on a spot plate and mix well. The visual detection of green–blue to blue color is to be regarded as a positive result indicating hematuria (Table 1).

2.3. In vitro studies

The following investigations were carried out to study in vitro interference from substances likely to be present in pathological urine samples, chloramphenicol; vitamin B complex; catalase; methyl iodide; methyl bromide; an aqueous suspension of lettuce (a peroxidase containing vegetable) and vitamin C for positive and negative results. The compounds studied are the common ingredients of a diet and or formulation(s) or supplementary formulation(s) generally prescribed by a physician to the patient suffering from the disorder. Further, the compounds chosen were presumed to affect the reaction.

To 0.2 ml of IPH or DPH reagent, 0.3 ml of glacial acetic acid and 0.1 ml of hydrogen peroxide solution, placed in a spot plate, were added: (a) 2–5 mg of powdered Enteromycetin (Dey's India), chloramphenicol (Dey's India), vitamin B complex, Cobadex forte (Glaxo India Ltd.), Macraberin forte (Glaxo India Ltd.) or catalase; or (b) 0.1–0.2 ml of Neurobion injection (Merck, India), Macraberin forte injection (Glaxo India Ltd.), methyl iodide (Sigma), methyl bromide (Sigma) or lettuce and mix. No blue color was seen even after 20 min. The test was repeated with 0.2 ml of oxidized IPH or DPH reagent. The blue color of the oxidized IPH or DPH did not disappear. These results suggest that the tested substances did not interfere, and gave neither false positive nor false negative results. The results were compared with the urine samples obtained from a healthy person who were kept on diet free of vitamin C, meat, fish, peroxidase containing vegetables and drugs. Thus 'healthy urine sample' is a reference to our studies.

When 0.1–0.2 ml of vitamin C (0.1% w/v) solution was added to the oxidized 0.2 ml of IPH or DPH reagent and 0.3 ml of glacial acetic acid, the blue color disappeared immediately, suggesting that the presence of vitamin C leads to false negative results. Moreover, the presence of upto 50 mg ascorbic acid per liter of urine did not interfere in the test. Various other reducing substances, like glucose, fructose, sucrose, urea, uric acid, creatine, creatinine, present in urine samples do not inhibit haematuria tests at concentrations upto 15 mmol/l.

2.4. In vivo studies

For in vivo tests each of three volunteers was given daily two tablets each of chloramphenicol and B-complex (250 mg) for 3 days. Their urines were tested using IPH and DPH reagents. The same volunteers were then given daily 1 g of vitamin C per person for 3 days, and their urines were tested using IPH and DPH reagents. In all cases, the results were the same as in the in vitro test; no interferences. However, results were less reliable in case of patients who were on 3 g or more ascorbic acid (Vitamin C) per day, due to poor reproducibility.

3. Results and discussion

Dibenzazepines are important tricyclic antidepressant drugs, having a central ring made up of seven atoms [22]. IPH and DPH belong to this important class of antidepressants, the efficacy of which has been well established [23].

The pseudoperoxidase activity of heme in haemoglobin, in an acetic acid medium, liberates nascent oxygen from hydrogen peroxide. The liberated oxygen oxidizes IPH or DPH and the details of the mechanism is described elsewhere [21].

3.1. Effect of metabolites and acid

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

IPH is metabolized primarily by two pathways: N-demethylation and hydroxylation [24]. 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-hydroximipramine, 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. Also, we studied the interference of tricyclic antidepressants such as amitriptyline, nortriptyline, doxepin and nordoxepin. No interference was observed.

Many experiments were carried out on the urine 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 behavior of the development of color 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 color. Thus, acetic acid medium has been selected for experiments.

3.2. Sensitivity and reproducibility

The results of our experiments with urine samples homogenized with human blood (obtained form blood bank) indicate that the intensity and the rate of development of color depend on the amount of blood present in urine. The sensitivity of IPH or DPH is 40 μl per 100 ml of urine. The standard deviation (S.D.) value of 3.9 indicates 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 color was seen even after 24 h. The results are presented in Table 1.

Table 2
Detection of hematuria in pathological human urine samples

Method	Negative	Trace	Positive (+)	Positive (2+)	Positive (3+)
Multistix	84	24	25	7	–
Benzidine	78	29	27	4	02
IPH	86	30	19	4	01
DPH	86	30	19	4	01

Number of samples, 140.

3.3. Stability

Solid IPH or DPH, if stored in a dry, brown bottle are stable for about 5 years. But, the solutions of IPH or DPH, prepared as described above, are stable for 2–3 months. The stability of the aforesaid solutions should not be confused with low stability of the oxidized IPH or DPH. The latter solutions were prepared to test in vitro inhibition of the color by reducing agents such as ascorbic acid.

One of the factors for low stability of the oxidized IPH or DPH is due to presence of large excesses of nitric acid which is an oxidizing agent.

3.4. Comparison of benzidine, multistix, IPH and DPH methods

Examination of urine samples for 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 the serious disadvantage of being a hazardous carcinogen.

One hundred and forty specimen samples of human urine, collected for tests in a leading hospital, were reacted with Multistix (Ames India Co.), benzidine, IPH, and DPH, respectively. Each sample was tested independently six times by the authors and two technicians in a hospital. The results are presented in Table 2. Positive 2+ and 3+ are more reliable data and the disorders have been confirmed by other methods, such as urine microscopy and urine cytology.

4. Conclusion

IPH and DPH are relatively inexpensive. They have required sensitivity and stability. The proposed reagents offer advantages of relatively low sensitivity to endogenous vegetable peroxidases; hence, the test can be performed on samples taken from patients who are on a normal diet and the results are reproducible. Besides, the reagents, are non-carcinogenic and can replace benzidine and *o*-tolidine without any extra procedural difficulties as they also exhibit a similar blue color.

Acknowledgements

The authors thank Ciba–Geigy, Switzerland for supplying IPH and DPH, respectively. The authors also thank Bhagwan Pathology Laboratory, Mysore and J.S.S. Hospital, Mysore for their assistance in carrying out clinical tests and University of Mysore, Mysore for granting permission to carry out research work.

References

[1] B. Bates, J. Mulinare, Physicians use and opinions of screening tests in ambulatory practice, J. Am. Med. Assoc. 214 (1970) 2173–2180.
[2] A.J. Mariani, M.C. Mariani, C. Macchioni, U.K. Stams, A. Hariharan, A. Moviera, The significance of adult hematuria: 1000 hematuria evaluations including a risk–benefit and cost effectiveness analysis, J. Urol. 141 (1989) 350–355.
[3] W. Catalona, Urothelial tumors of urinary tract, in: P. Walsh, A. Retik, T. Stamey, J. Vaughan (Eds.), Campbell's Urology, Saunders, Philadelphia, PA, 1992, p. 1094.

[4] J. Dekernion, A. Belledegrun, Renal tumors, in: P. Walsh, A. Retik, T. Stamey, J. Vaughan (Eds.), Campbell's Urology, Saunders, Philadelphia, PA, 1992, p. 1053.
[5] E.M. Messing, A. Vaillancourt, Hematuria screening for bladder cancer, J. Occup. Med. 32 (1990) 838–841.
[6] J.P. Arm, E.B. Peile, D.J. Rainford, P.W. Strike, R.E. Tettmar, Significance of dipstick haematuria 1. Correlation with microscopy of the urine, Br. J. Urol. 58 (1986) 211–217.
[7] A. Asberg, E.H. Lehmann, J.E. Petterson, Evaluation of dipsticks for the assessment of microhematuria, Scand. J. Clin. Lab. Invest. 44 (1984) 377–380.
[8] J.M. Budinger, M. Cavallo, Detection of hematuria with a paperstrip indicator, Am. J. Clin. Pathol. 42 (1964) 626–629.
[9] A.P. Goldner, R. Mayron, E. Ruiz, Are urine dipsticks reliable indicators of hematuria in blunt abdominal trauma patients?, Ann. Emerg. Med. 14 (1985) 580–582.
[10] J.A. James, N.H. Liebschutz, Evaluation of a new 'dip and read' test for hematuria, Am. J. Clin. Pathol. 38 (1962) 554–556.
[11] M. Bradley, G.B. Schumann, P.C.J. Ward, Examination of urine, in: J.B. Henry (Ed.), Clinical Diagnosis and Management of Laboratory Methods, Saunders, Philadelphia, PA, 1979, p. 559.
[12] M.S. Litwin, S.D. Graham, False-positive hematuria, J. Am. Med. Assoc. 254 (1985) 1724–1728.
[13] M.R. Weaver, I. Gib, Urinalysis for blood—questionable interpretation of reagent strip results, Clin. Chem. 29 (1983) 401–402.
[14] G.B. Schumann, S.C. Schweitzer, Examination of urine, in: J.B. Henry (Ed.), Clinical Diagnosis and Management by Laboratory Methods, Saunders, Philadelphia, PA, 1991, p. 412.
[15] J.P. Gregerson, Studies on occult blood, Arch. Verdauungs-Krankh 25 (1919) 169–175.
[16] C.E. Searle, Chemical carcinogens and their significance for chemists, Chem. Britain 6 (1970) 5–10.
[17] V.R. Holland, B.C. Saunders, F.L. Rose, A.L. Walpole, A safer substitute for benzidine in the detection of blood, Tetrahedron 30 (1974) 3299–3302.
[18] S.A. Ahmed, H.S. Gowda, Using phenothiazines to detect micro amounts of blood in urine, Clin. Chem. 27 (1981) 1309–1310.
[19] H. Caplan, G. Discombe. On chemical tests for blood in urine, Br. Med. J. 4 (1951) 774–775.
[20] M.A. Sinatra, D. James, J. St, G.P. Young, Interference of plant peroxidases with guaiac-based fecal occult blood tests is avoidable, Clin. Chem. 45 (1999) 123–126.
[21] A.A. Syed, B.A. Khatoon, M.F. Silwadi, New reagents for detection of faecal occult blood, J. Pharm. Biomed. Anal. 24 (2001) 581–586.
[22] A. Burger (Ed.), Medicinal Chemistry, Part II, Third ed., Wiley-Interscience, New York, 1970, p. 1475.
[23] R.F. Doerge (Ed.), Wilson and Gisvolds Text-book of Organic Medical and Pharmaceutical Chemistry, eighth ed., Lippnicott, Philadelphia, PA, 1982, p. 392.
[24] D.J. Sequeria, H.W. Strobel, High-performance liquid chromatographic method for analysis of imipramine metabolism in vitro by liver and brain microsomes, J. Chromatogr. B 673 (1995) 251–258.