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COMPREHENSIVE REVIEW OF IMPORTANT ANALYTICAL REAGENTS USED IN SPECTROPHOTOMETRY

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ABSTRACT

An innovative and comprehensive range of chromogenic reagents or colorimetric reagents are generally used for the application in the field of spectroscopic quantitative and qualitative analysis. There are number of reagents which are mostly utilized for the estimation of pharmaceutical medicinal agents. Functional groups generally present in medicinal drugs determine the way of analyzing them, because they are responsible for the properties of substances and estimate the identification reactions and the methods of quantitative determination of medicinal agents. Knowing the reactions for detecting functional groups, one can easily analyze any medicinal agents with complicated structure. This Reagents review provides information about the different reagents used in the estimation of the compounds by colorimetric methods. In this review reagent preparation, some reaction mechanisms, handling procedures and applications are discussed. This entire review covers the Bratton-Marshall (BM) [Diazotization followed by coupling], 3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride (MBTH) [oxidative coupling reaction], Para Dimethyl Amino Benzaldehyde (PDAB) and Para Dimethyl Amino Cinnamaldehye (PDAC) [condensation reactions with aromatic aldehydes], Folin-Ciocalteu (FC) [oxidation/reduction], 1,2-Naphtha Quinone-4-Sulphonate Sodium (NQS reagent), 2,6-Dichloro quinone-4-Chloroimide **(**Gibb's reagent), Bathophenanthroline, 1,10-phenanthroline, 2,2'-Bipyridine [oxidation followed by complex formation], METOL reagents [oxidation followed by charge transfer reaction]. In this review, we decorously summarized and discussed the important analytical chromogenic reagents regularly used in drug analysis by visible spectrophotometric methods. All above listed regents are most extensively used in the estimation of several pharmaceutical compounds. These reagents have a number of applications in the pharmaceutical field and also applied to novel analytical techniques.

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INTRODUCTION

The most commonly employed reagents for the determinations are the following:

- *N*-1-Naphthyl ethylene diamine dihydrochloride called as Bratton–Marshall reagent (BM reagent) undergoes diazotization for the determination of sulpha drugs, local anaesthetics, etc.
- 3-Methyl-2- benzothiazolinone hydrazone hydrochloride, which is generally known as MBTH. The color production is depending upon the oxidative coupling of this reagent with phenols, amines, carbonyl compounds, etc.
- Para dimethyl amino benzaldehyde (PDAB) and para dimethyl amino cinnamaldhyde (PDAC) - certain amines condenses with a variety of aldehydes in acidic media to give a product which are colored and oxidisable.
- Phosphomolybdotungstic acid called as Folin – Ciocalteu reagent (FC reagent) undergoes oxidation, reduction (or) hydrolysis based on the functional group to be determined such as amines, phenols etc.
- 1,2-Naphthoquinone-4-sulfonate sodium (NQS) is a chromogenic agent used for the determination of 1° aromatic amines.
- 2,6-Dichloroquinone chloroimide, which is commonly known as GIBB'S reagent used for the identification and estimation of phenols.
- Oxidation followed by complexation. Ex: Bathophenanthroline, 1,10 - phenanthroline, 2,2'-Bipyridine.
- Oxidation followed by charge transfer complex formation ex: Metol-KIO_3.

BRATTON–MARSHALL REAGENT (BM REAGENT)

It is chemically *N*-1-naphthyl ethylene diamine dihydrochloride [(1-5)]. It is extremely sensitive and extensively utilized chromogenic reagent for the determination of drugs and pharmaceuticals containing free primary aromatic amino group. At present, it is used for the determination of compounds containing primary aromatic amino group as well as compounds which produce primary aromatic amino group up on chemical modification such as reduction of nitro group and hydrolysis of carboxamido group. Some of the classical examples include local anesthetics, sulfa drugs. The chemical structure of Bratton–Marshall reagent is shown in Fig 1.

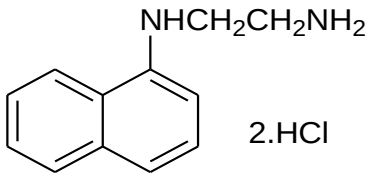


Figure 1. Structure of N-1-Naphthyl ethylene diamine dihydrochloride.

Reaction and mechanism

The primary aromatic amino group is first diazotised with sodium nitrite and hydrochloric acid. The excess nitrous acid (HNO_2) is neutralized by treating with ammonium sulphamate reagent. Finally, the diazonium ion is allowed to couple with Bratton-Marshal reagent (NED) to produce a highly colored azo dye complex (Fig 2) measured at 550 nm.

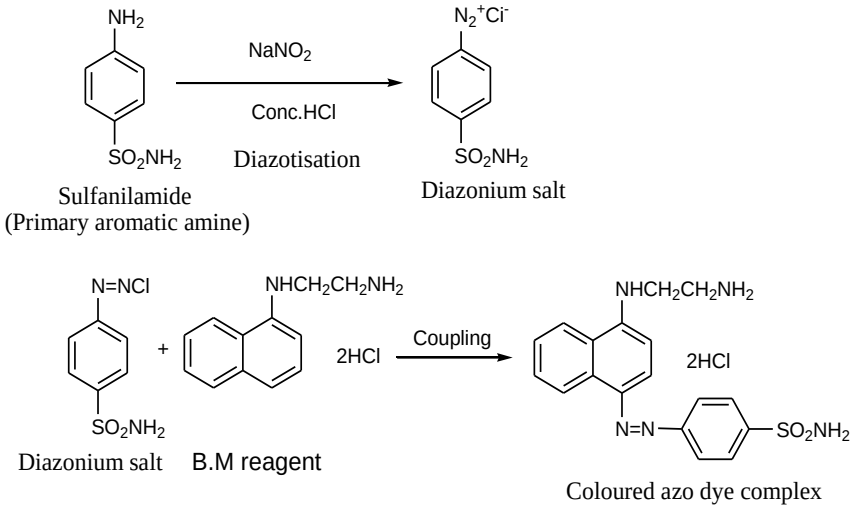


Figure 2. Formation of colored azo dye complex.

First, nitrous acid ($NaNO_2$/HCl) furnishes nitrosonium ions $[N=O]^+$. Then nitrosonium ions combine with sulfanilamide to form nitroso sulfanilamide derivative, which undergoes rearrangement and eventual dehydration to form diazonium ion which take up Cl^- to yield the diazonium salt (fig 3). The diazonium salt undergoes azo coupling reaction with B.M reagent by following electrophillic aromatic substitution reaction, where the diazonium salt acts as the electrophile. The substitution occurs in the para position of B.M reagent due to the fact that the other position is sterically hindered. Eventually this solution is allowed to couple with B.M reagent to produce a highly pink colored azo dye complex (fig 4).

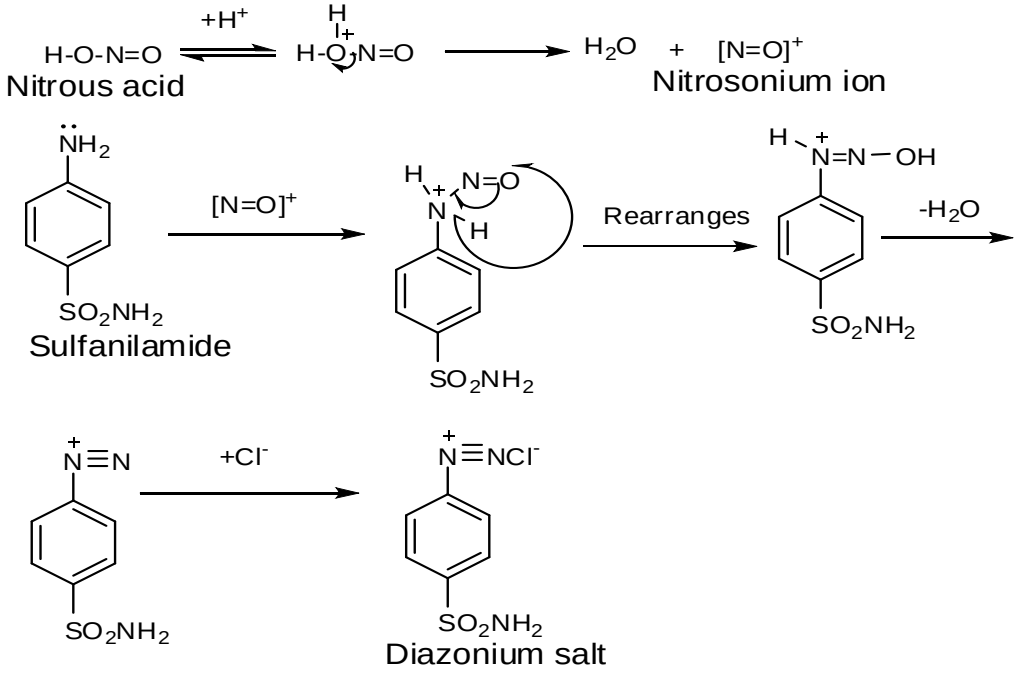


Figure 3. Formation of diazonium salt.

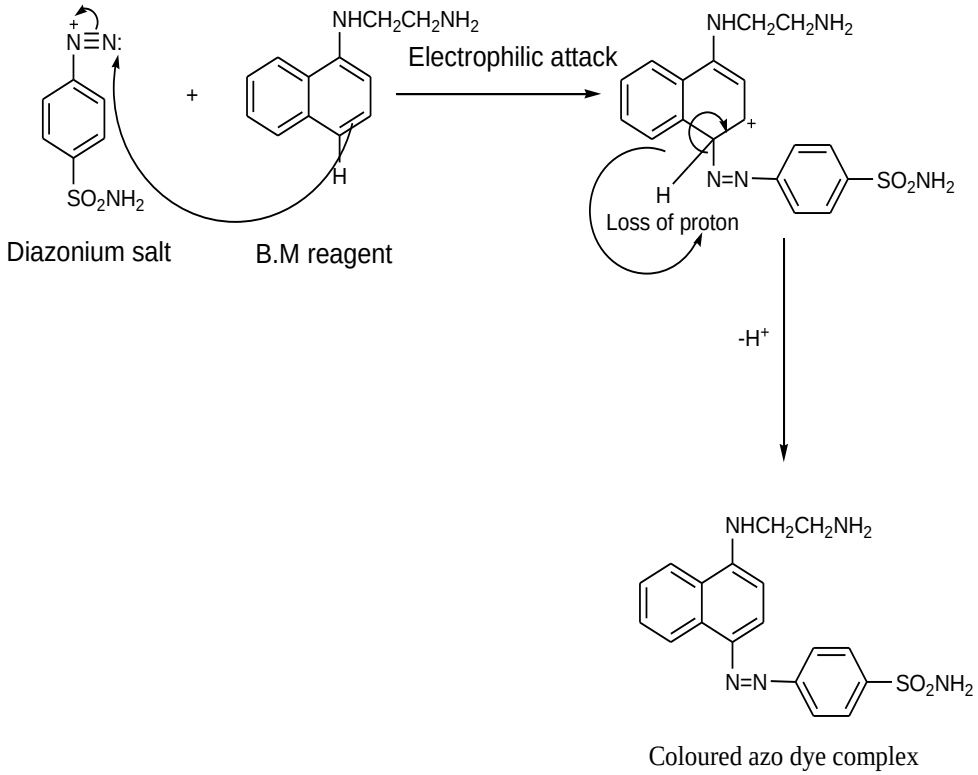


Figure 4. Reaction mechanism involved in the formation of pink colored azodye complex.

Preparation of B.M reagent:

Dissolve 100 mg of *N*-1-naphthyl ethylene diamine dihydrochloride in 100 ml of a mixture of seven parts of acetone and three parts of water.

Applications of B.M reagent:

B.M reagent is one of the most widely employed chromogenic reagent for the estimation of a variety of therapeutic classes of drugs by colorimetrically (or) visible spectrophotometrically. The drugs or pharmaceuticals are estimated by either direct or indirect methods.

Direct method:

It involves direct reaction of drugs containing primary aromatic amino groups to form diazonium chloride. Later the diazonium chloride is coupled with B.M reagent to form colored azo dye complex.

Indirect method:

In this method the drug molecules are chemically modified to generate primary aromatic amino groups either by reducing the aromatic nitro group (fig 5) or by hydrolyzing amido group. Drugs estimated by direct and indirect method by using B.M reagent are shown in table 1. Table 2 shows the list of drugs analyzed with BM reagent in direct method in UV.

Table 1. Drugs estimated by direct and indirect methods by using B.M reagent.

S. No	Name of the drug	Class
DIRECT METHOD (Drugs with intact primary aromatic amino group)		
1	Dapsone	Antileprotic
2	Sulfadiazine, sulfanilamide, sulfaphenazole, sulfamethoxazole, sulfadimidine, sulfathiazole, sulfaguanidine, sulfacetamide sodium, sulfadimethoxine.	Antibacterial agents
3	Para-aminosalicylic acid	Antitubercular
4	Benzocaine, procaine	Local anesthetic
INDIRECT METHOD (Drugs which produce primary aromatic amino group on reduction)		
1	Metronidazole, tinidazole	Antiamoebic agents
2	Chloramphenicol	Antibiotic
3	Nifedipine	Antihypertensive
4	Nitrazepam	Hypnotic
5	Nimesulide	NSAID
6	Paracetamol*	Analgesic-antipyretic
7	Succinyl sulfathiazole*, Pthalyl sulfathiazole*	Antibacterials

* = Drugs which produce primary aromatic amino group after hydrolysis.

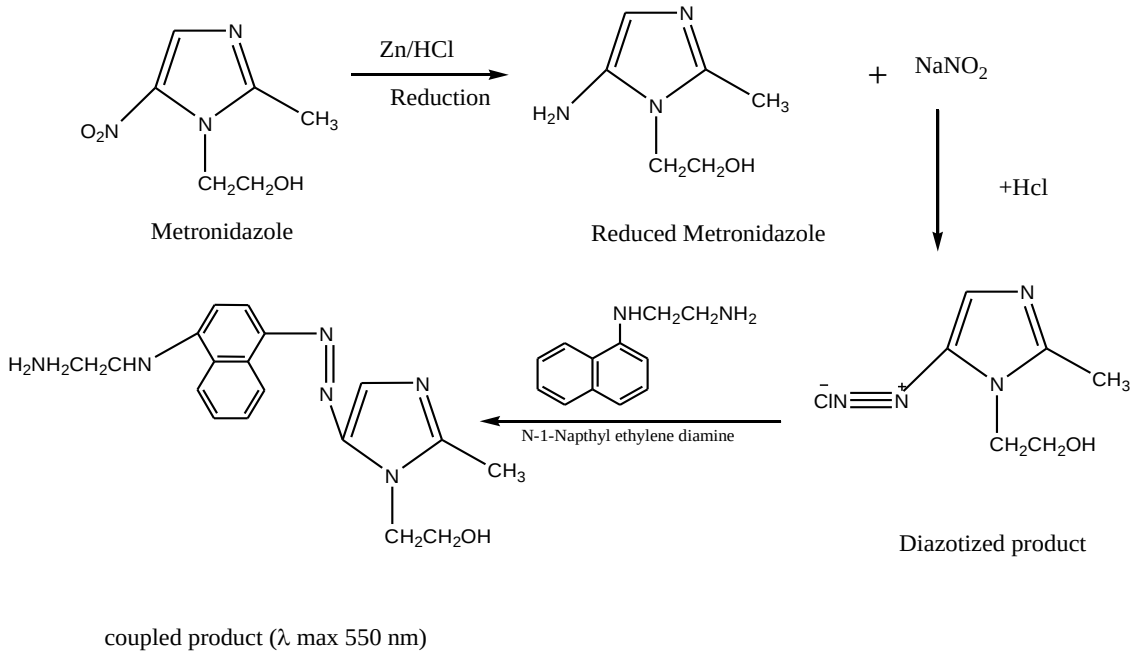


Figure 5. Metronidazole reaction with BM reagent.

Table 2. List of drugs analyzed with BM reagent in direct method in UV.

S.No	Drug	Method	λ_{max} (nm)	Beers law limit (µg/ml)
1	Lenalidomide	Direct	540	1-5
2	Pramipexole	Direct	616	5-25
3	Sulfacetamide	Direct	530	1-3
4	Amisulpride	Direct	530	1-5

3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride (MBTH) (Besthorn's reagent or Sawicki's reagent)

3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride (MBTH) [6-14] was initially synthesized by Besthron in 1910. Huning and Fritsch have described the oxidative coupling of this reagent with phenols, aromatic amines, heterocyclic bases and compounds containing active methylene group to form extremely colored products in 1957. In 1961 Sawicki et al. introduced MBTH in analytical chemistry as a sensitive reagent for the determination of carbonyl compounds. It can be also utilized for the detection and determination of phenols, polyhydroxy compounds, aldehydes, aromatic amines and amino hetero aromatic compounds including indoles, carbazoles and phenothiazines. The chemical structure of MBTH is shown in Fig 6.

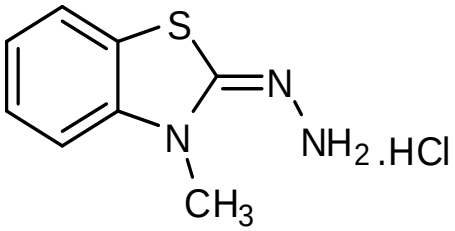


Figure 6. 3-Methyl -2-Benzothiazolinone Hydrazone Hydrochloride (MBTH).

General mechanism for the reaction of MBTH

MBTH loses two electrons and one proton on oxidation, under reaction conditions, forming an electrophilic intermediate, which is assumed to be the active coupling species. The intermediate reacts with amine (or) phenol by electrophilic attack on the mainly nucleophilic site up in the aromatic ring of amine or phenol (i.e., para or ortho position) and the intermediate is oxidized in the presence of oxidant to get the colored species. Fig 7 shows the general reaction with MBTH.

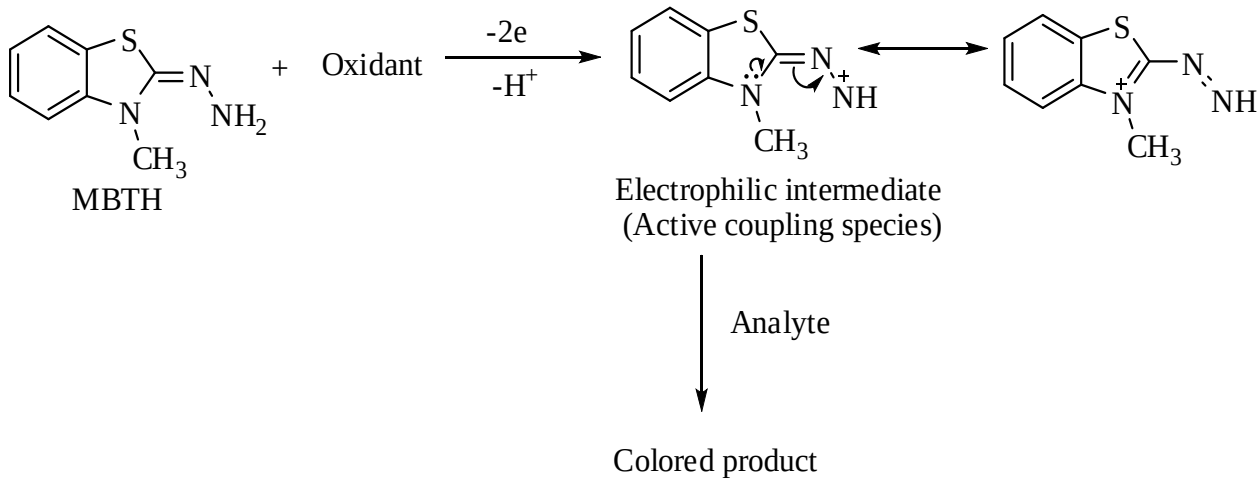


Figure 7. General reaction with MBTH.

Principle of MBTH reagent:

3-methyl-2-benzothiazolinone hydrazone hydrochloride originally introduced as a reagent for aldehydes. Later MBTH use was extended to different organic compounds (example: Phenols, Aryl amines and different N- and S- heterocyclic compound). MBTH reacts with aldehyde first to form an azine. Only if there is remaining MBTH, it is oxidized to another species which combines with the azine to form formazan. However, if there is enough aldehyde, all the MBTH is converted to azine and there is no formation of blue color. Thus, by using the limiting agent MBTH to test the amount of aldehyde around the point of interest, then less aldehyde would produce extra blue color and more aldehyde would produce less blue color. The end color may be different depending upon the order of addition of the reactants. For example, if an oxidizing agent and MBTH are mixed before adding the aldehyde, a light green to green/blue color results. Fig 8 shows the formation of formazan with MBTH.

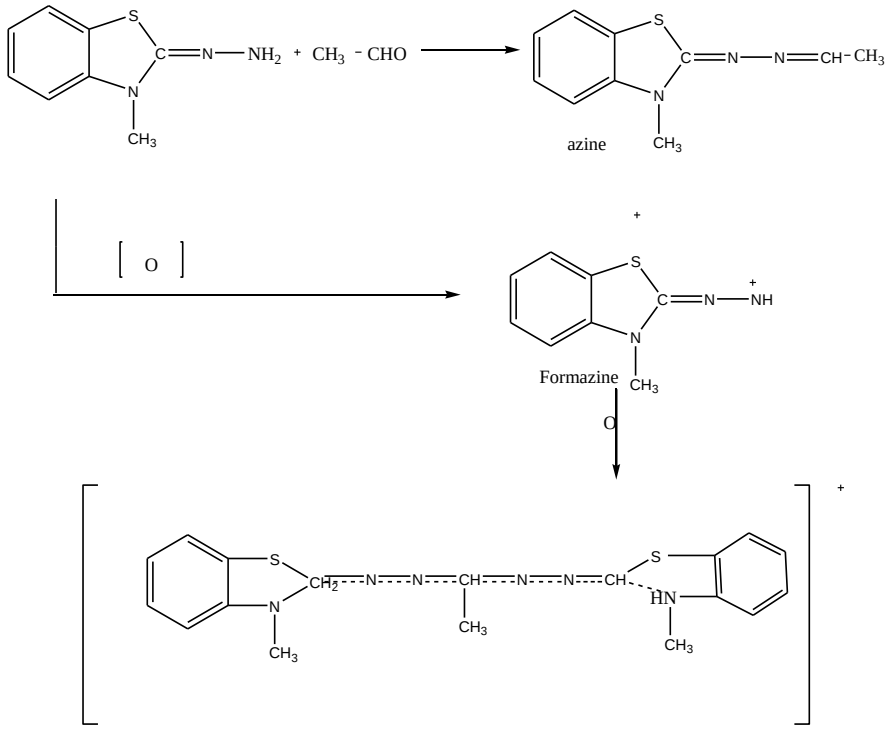


Figure 8. Formation of formazan with MBTH.

I. Reaction with phenols:

The reaction of MBTH with phenols is carried out in acidic, alkaline (or) neutral medium and in the presence of oxidizing agents by oxidative coupling. The colors obtained with phenols. Generally, co-substituted phenols get orange to red and Alkyl-constituted phenols get violet (or) less intense color. As said by Hunning and Fritsch, phenol reacts in the para position to the hydroxy group which is to some extent common in oxidative coupling reactions. Pays and Bourdon and Wanda subjected the colored reaction products to thin layer chromatography (TLC) and observed that phenol and *ortho* and *meta* cresols gave single red sports. Fig 9 shows the MBTH condenses blue cation.

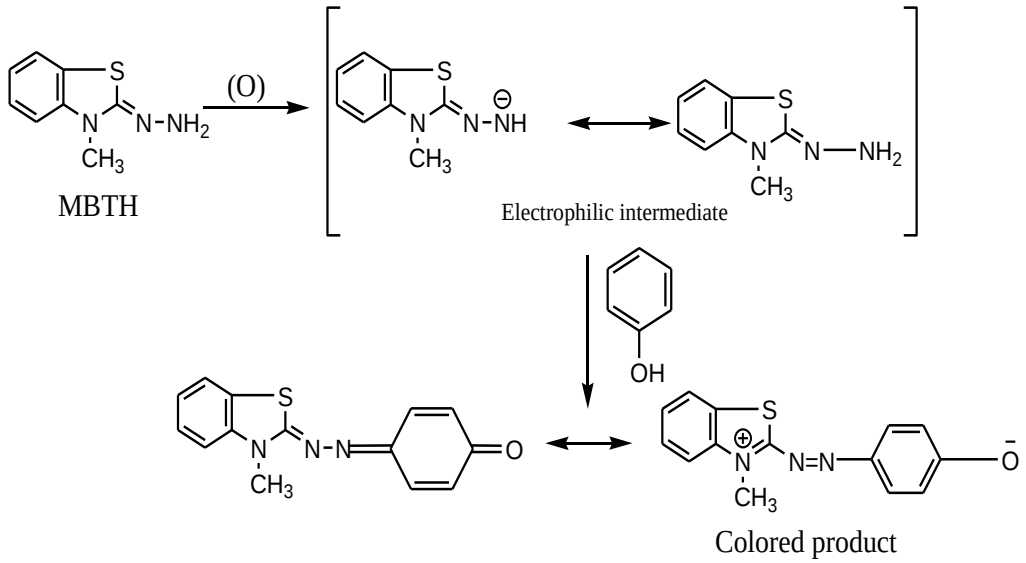


Figure 9. MBTH condenses blue cation.

II. Reaction with amines:

Sawicki et al. investigated the reaction of MBTH with a number of amines. They found that the reagents reacts readily with most aromatic amines and forms an azodye cation. It was reported that the reagent probably attacking the amines mainly in the para position or the ortho position if the para position is not free. The mechanism of reaction is shown in fig 10.

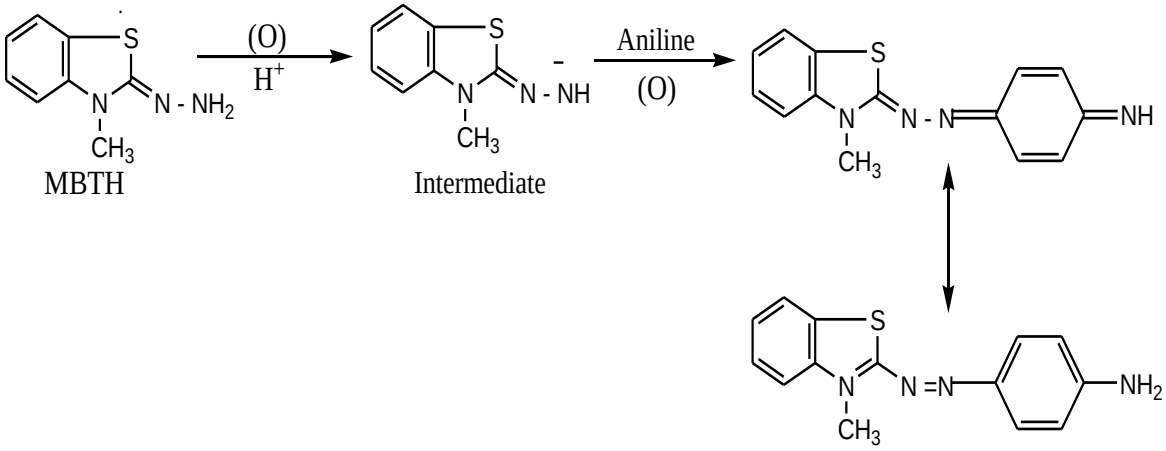


Figure 10. Reaction with amines.

III. Reaction with aldehydes:

Reaction of formaldehyde and other aldehydes with MBTH was investigated by Sawicki et al. Reaction HCHO with MBTH to form azine and MBTH undergoes oxidation to form an electrophilic intermediate which reacts with the azine and forms blue cation (fig 11).

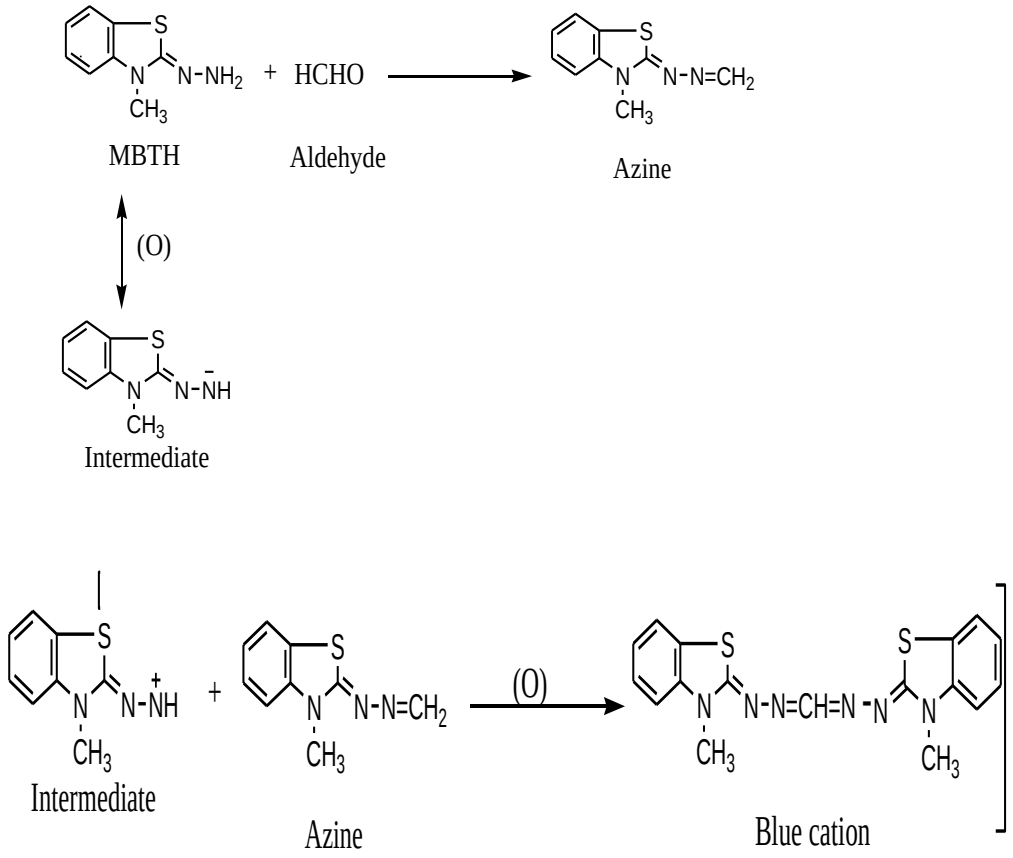


Figure 11. Formation of blue cation with MBTH.

Oxidizing agent used for color production with MBTH

The following oxidizing agents are employed:

- Ferric chloride
- Cerric ammonium sulphate
- Sodium meta periodate
- Potassium dichromate
- Potassium bromate
- Ammonium per sulphate
- Ferric cyanide, etc.

The most widely used oxidants among the above are cerric ammonium sulphate and ferric chloride.

Factors affecting the nature and intensity of color

Generally five factors affect the nature and intensity of the resulting colored choromogen include

- Reagent (MBTH) concentration.
- pH of the medium.
- Temperature.
- Nature and concentration of oxidizing agent.
- Order of addition of reagents.

Applications of MBTH

- Less time is required for production of color.
- It is utilized for the determination of drugs at very small concentrations, in biological fluids such as blood and urine.
- It is applicable to determination of drugs like acyclovir, ganiclocir, ceftazidine, cefradoxil and nicorandil.
- Utilized in the identification of aldehyde, amines, phenols, aryl amines.
- It is applicable to analysis of drinking water, domestic and industrial wastes.
- MBTH is utilized for estimation of samples which contain higher concentrations of aldehyde like disinfectants.

Table 3. List of pharmaceuticals estimated with MBTH reagent.

Name/type of the compound	Oxidant used
Phenylephrine, thymol, salicylic acid, adrenaline, noradrenaline, bisacodyl, enrofloxain, ofloxacin, isoxsuprine, nylydrine, pholedrine, salbutamol, amidopyrine, paracetamol, epinephrine, isoprenaline, amisulpride.	Ceric ammonium sulfate
Naproxen, pentazoline, mefenamic acid, analgin, primaquine, cortisone, prednisolone, nitrozepam (reduction), ephedrine, pyridoxine, riboflavin, glucose in serum (or) plasma.	Ferric chloride
Methyl dopa, dopamine.	Potassium dichromate
Phendione (1-phenyl pyrazolidin-3-one).	Ammonium per sulphate
Heparin	Periodate

MBTH reagent has different applications in the determination and spectrophotometric estimation of various drugs (Table 3) in pure samples, dosage forms and in biological fluids. Fig 12 shows the Amesulpride reaction with MBTH.

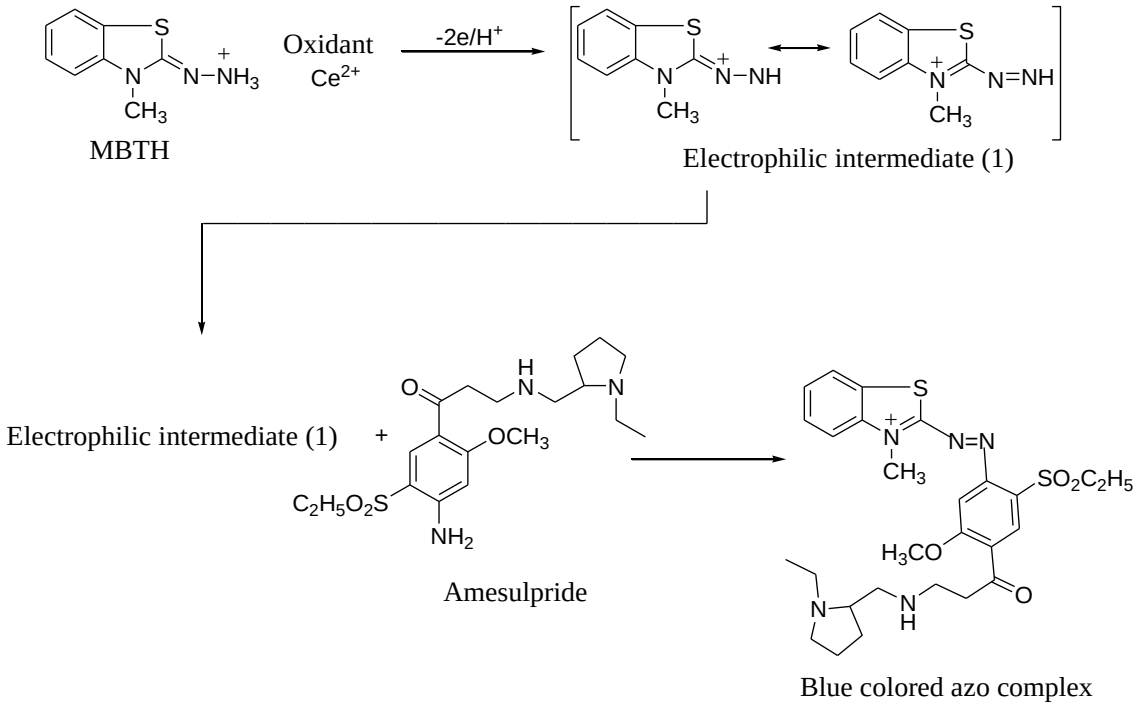


Figure 12. Amesulpride reaction with MBTH.

PARA DIMETHYLAMINO BENZALDEHYDE (PDAB), (DMAB or Ehrlich's reagent)

PDAB (Ehrlich's reagent) is most widely employed chromogenic reagent used for the colorimetric determination of a number of drugs or pharmaceutical substances containing aromatic amino group**.** PDAB reagent [15-17] is generally used for various drugs containing the $-NH_2$ group. The chemical structure of PDAB is shown in fig 13.

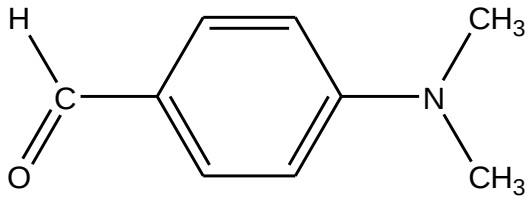


Figure 13. Structure of PDAB.

Preparation of PDAB solution:

It is prepared by dissolving 2.0 g of P–Dimethylaminobenzaldehyde (PDAB) in 50 mL of 95% ethanol and 50 mL of concentrated hydrochloric acid. It must prepared fresh before using.

Reaction mechanism:

The mechanism of aldehydes which condenses with the aromatic amines involves the condensation of the aldehydes to release the oxygen molecule and then it combines with the amine group to form the yellow Schiff's base in the presence of acidic medium such as HCl or H_2SO_4. Fig. 14 shows the formation of schiff's base.

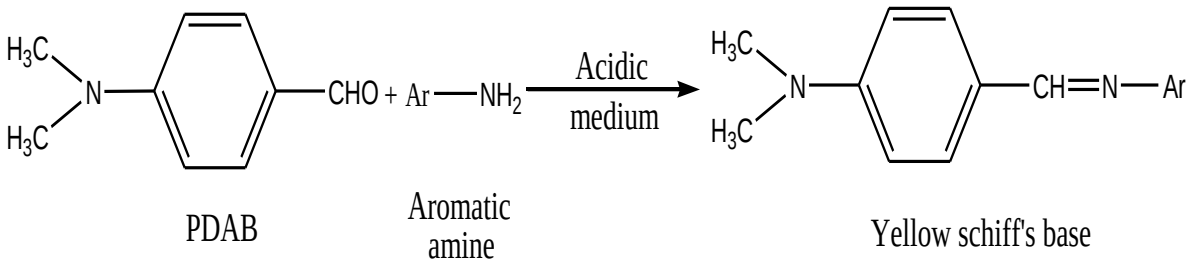


Figure 14. Formation of yellow Schiff's base.

Applications:

Determination of terbutaline and orciprenaline:

Preparations:

Dissolve 125 mg of reagent (PDAB) in a cooled mixture of 65 ml of sulphuric acid, 35 ml of water and 0.05 ml of ferric chloride which should be used within one week.

Method with PDAB:

Sample solution is mixed with the equal amount of PDAB reagent and metabolic sulphuric acid. The solution is kept aside for the color production. The yellow colored Schiff's base is measured at 450 nm.

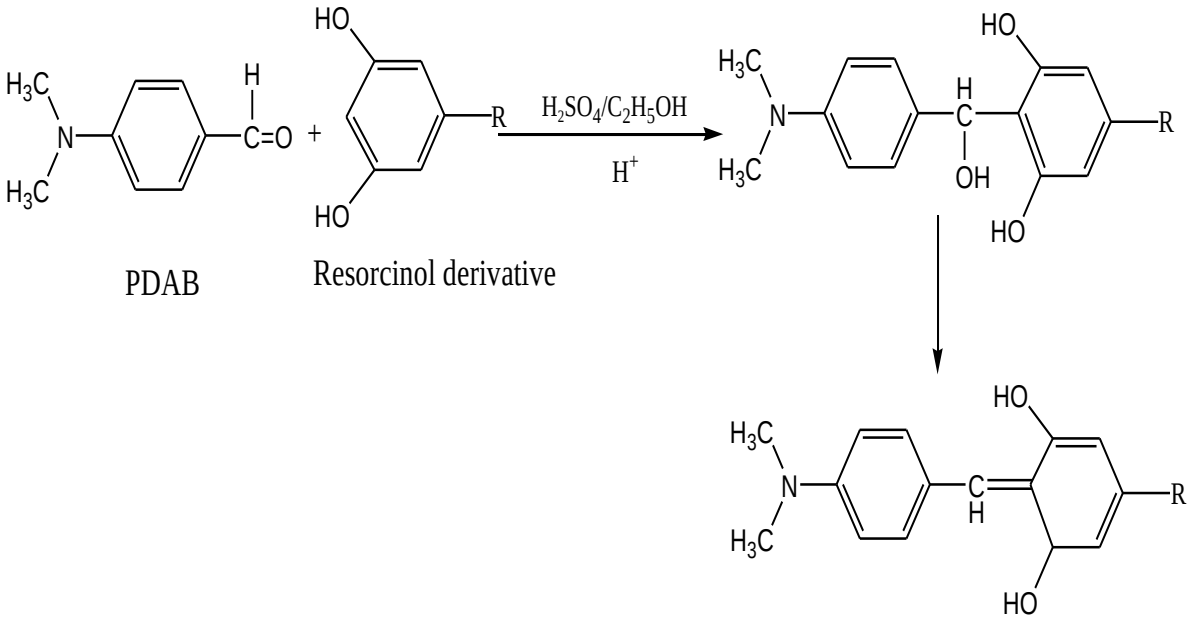


Figure 15. Formation of Schiff's base.

Determinations of Pyrroles and Indoles with PDAB (Ehrlich reaction):

Ehrlich found that the PDAB condensed with pyrroles in an acidic medium generate a red-violet color. The colored product is a quinine amine. The reaction is shown in fig 16.

For Pyrroles

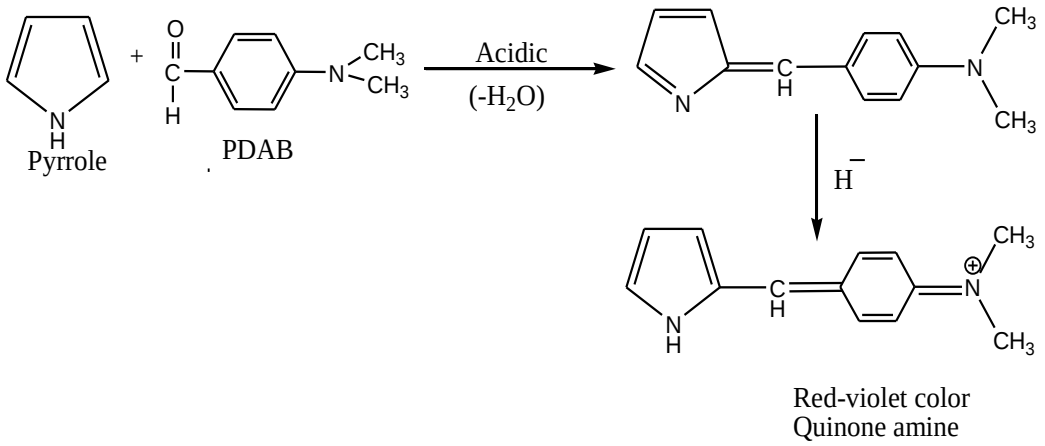


Figure 16. PDAB reaction with Pyrrole.

For Indoles

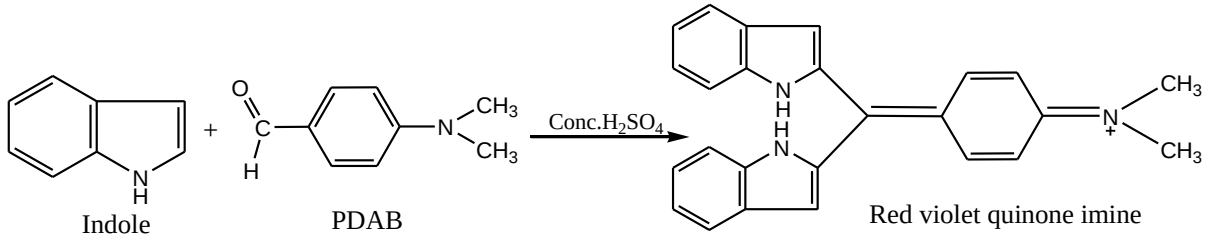


Figure 17. PDAB reaction with indole.

Examples of drugs estimated by Ehrlich reaction are dihydroergotamine and methyl ergotamine, which are estimated by PDAB in the presence of 6M H_2SO_4 heated at 25–50 °C for 60 min.

Estimation of Sparfloxacin:

Sparfloxacin undergoes condensation with PDAB under acidic conditions to produce yellow color Schiff's base with absorption maxima of 455 nm.

Hydrazine estimation:

PDAB reacts with hydrazine to form an azo dye, which has a distinct yellow color. It is therefore utilized for spectrophotometric determination of hydrazine in aqueous solutions at 457 nm. Fig 18 and Fig 19 shows the PDAB reaction with Ganciclovir and Sulphamethoxazole respectively. List of drugs, estimated with PDAB reagent is shown in Table 4.

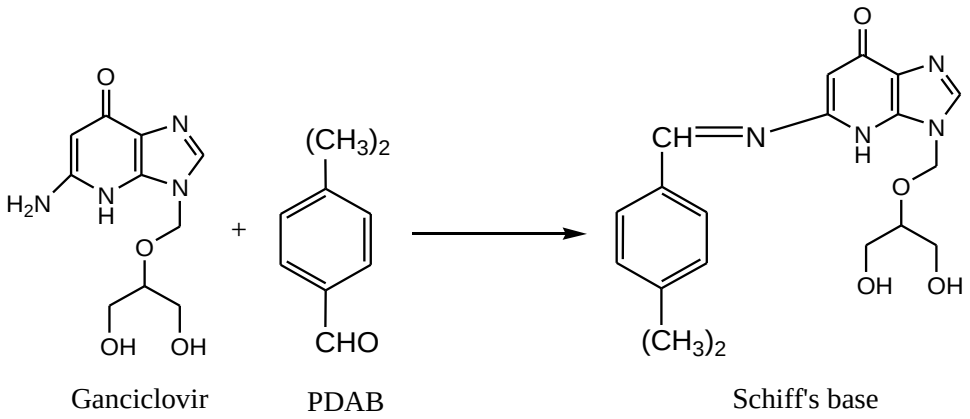


Figure 18. PDAB reaction with Ganciclovir.

Table 4. List of drugs estimated with PDAB reagent.

	Names of the drugs react with PDAB
1	Sulfadiazine, sulfisoxazole, sulfamethoxazole, sulfathiazole, sparfloxacin, sulfamerazine, analgin, caffeine.
2	After reduction Chloramphenicol, succinyl sulphathiazole, nimesulide, nitrendipine, nimodipine, nifedipine.

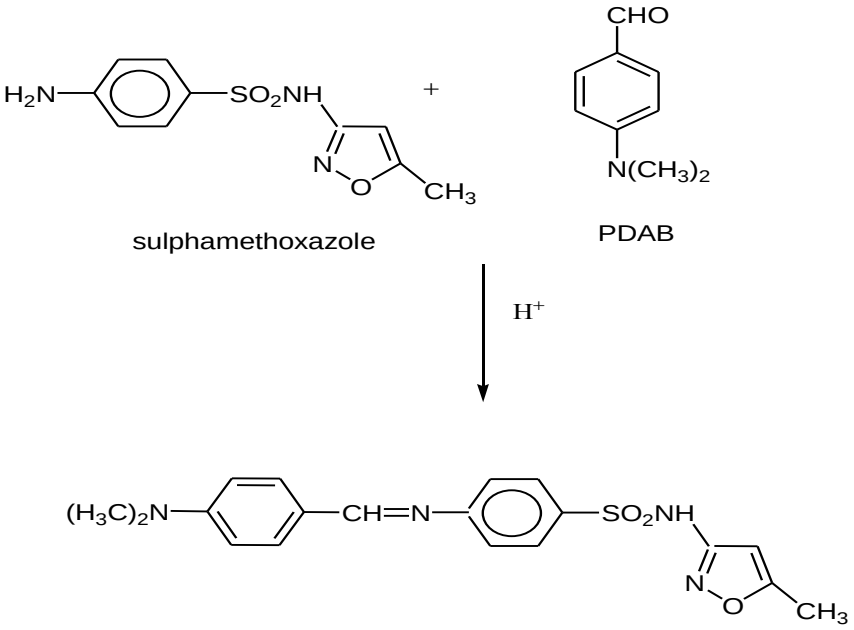


Figure 19. Yellow colored Schiff's base with PDAB.

PARA DIMETHYL AMINO CINNAMALDEHYDE (PDAC) (or) (DMACA REAGENT)

Certain amines condense with different aldehydes in strong acidic media to give products that are oxidisable to give a color. Among many aldehydes that have been to shown to react is p-dimethylamino cinnamaldehyde [18-20] which give most excellent results. The most common oxidant utilized is atmospheric oxygen, but the process has been hastened by the addition of H_2O_2, nitrates, ferric iron and several other metal ion catalysts. In general, drugs containing aromatic amino group or generating those groups by reduction or hydrolysis are determined by making use of this reagent. 4-Dimethyl amino cinnamaldehyde is a chromogenic reagent for indoles and flavanols. Chemical structure of PDAC is shown in fig 20. It is used to estimate the ability of an organism to split indole from the tryptophan molecule. The empirical formula of DMACA reagent is $C_{11}H_{13}NO$.

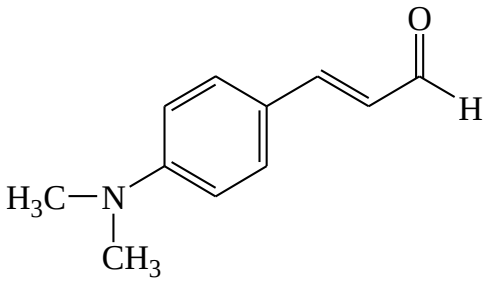


Figure 20: Structure of PDAC.

Solution composition of PDAC per 100 ml.

P- Dimethyl amino cinnamaldehyde -1 gm.
Hydrochloric acid (concentrated) - 1.0 ml
Distilled water - 99.0 ml

Method with PDAC:

The sample is mixed with the PDAC reagent and then slowly the ethanolic sulphuric acid is added and allows standing for the color production. The resulting yellow color Schiff's base is measured at 455 nm, which is shown in fig 21.

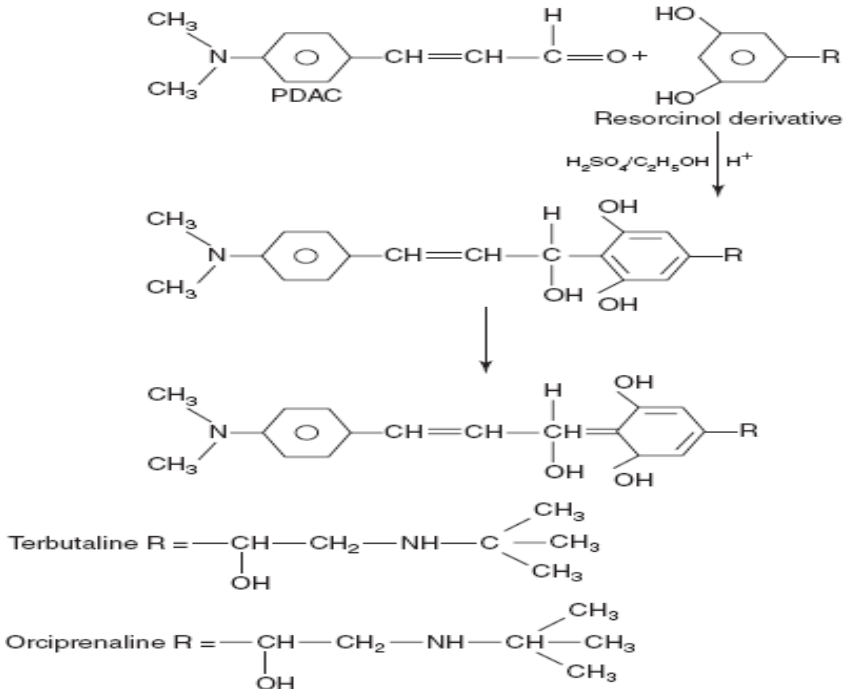


Figure 21: Formation of yellow colored Schiff's base.

Applications:

- Used to estimate the ability of an organism to split indole from the tryptophan molecule.
- Reagent for the rapid identification of Streptococci.
- Reagent for assay of indole product of apotryptophanase and tryptophanase.
- PDAC produces colored adducts with flavanols for subsequent HPLC.
- It has been used in a chromogenic method for quantifying proanthocyandines in a cranberry powder.
- In microbiology, DMACA disks and strips are utilized in the identification and confirmation of microorganisms. They are simple to use and based on rapid screening methods like the detection of enzymes with chromogenic substrates, indicators, or on complex building reactions. Also, the sensitivity to certain inhibitory substances can be utilized to identify organisms. The sterility indicator strips are utilized to monitor sterilization and are vital for controlling the sterilization process.
- Indole reagent (1% cinnamaldehyde) is the reagent of choice for performing the spot indole test. The spot indole test is a fast procedure designed by Vracko and Sherris. It can be utilized to presumptively characterize Escherichia coli, and to differentiate swarming proteus species.
- Indole reagent (PACA) is the normally sensitive reagent and can detect as little as 3.0 µg of indole per mL. It has also been found helpful for examining anaerobic organisms.
- DMACA Indole disks are utilized for indole test to estimate the ability of an organism to split tryptophan into indole and α-aminopropionic acid. The existence of indole can be detected by the addition of DMACA which results in a bluish-purple complex. With this method, it is possible to differentiate *Escherichia coli* from *Klebsiella*.

To aid in differentiating between genera:
1. Separate Escherichia coli (+) from members of Klebsiella (V-), Enterobacter (V-), Hafnia (-), Serratia (V-) and Pantoea (-).

To aid in differentiating between species:

- Paenibacillus alvei (+) from other Bacillus spp. (-)
- E. Coli (+), E. Hergusonii (+), E. Hermanii (+), from E. Vulneris (-), E. Blattae (-)
- Proteus vulgaris (+), P. Inconstans (+), P. Rettgeri (+) from other Proteus spp.
- Klebsiella oxytoca (+), K. Ornithinolytica (+) from other Klebsiella spp. (usually).

Uses:

- Along with other tests (urease and ornithine) subdivides Haemophilus influenzae, Haemophilus parainfluenzae into biotypes.
- Along with sialidase, α and β-glucosidase, α-fructosidase, differentiates between black-pigmented anaerobes: Porphyromonas asaccharolyticus (+), P. Ndodontalis (+), P. Gingivalis (+), and Prevotella intermedia (+) from Prevetolla corporis (-), P. Denticola (-), P. Loesheii (-), P. Melaninogenica (-) and Porphyromonas Levii (-).

Interpretation of Results:

Positive : Blue to blue-green color (within 30s).

Negative : Colorless or light pink.

Storage and Shelf Life:

Indole reagent should be stored at 4°C to 8°C and protected from light. Under these conditions, it has a shelf life of 26 weeks from the date of manufacture.

Table 5. List of drugs estimated with PDAC.

S.NO	Reagent used	Drug analysed
1.	PDAC	Nimesulide, nifedipine, nimodipine, chloramphenicol (After reduction)

Estimation of nimesulide:

Nimesulide, *N*-[4-nitro-2-phenoxypheny] methane sulphonamide estimation is based on converting nimesulide to its reduced form by zinc dust and HCL. One aromatic amino group is reduced. The resulting red colored solution is measured at 520 nm. Table 5 shows the list of drugs, estimated with PDAC.

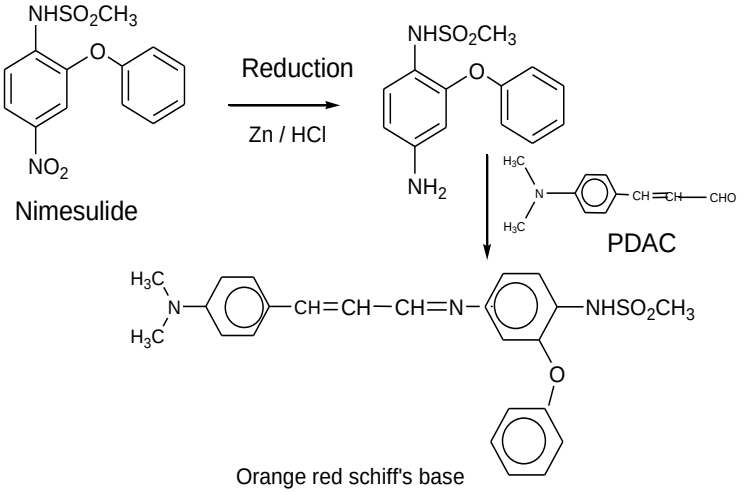


Figure 22. Formation orange colored schiff's base.

FOLIN–CIOCALTEU REAGENT (FC REAGENT) (OR) FOLIN'S PHENOL REAGENT (OR) FOLIN-DENIS REAGENT

Folin–Ciocalteu reagent was first introduced by O. Folin and D. Ciocalteu in 1927. Chemically, it is a heteropolyacid that is Phosphomolybdotungstic acid. The Folin-Ciocalteu reagent (FCR) [21-29] is also known as Folin's phenol reagent or Folin-Denis reagent. The assay method used, this reagent is known as "Gallic Acid Equivalence method" (GAE), which contains mixture of phosphomolybdate and phosphotungstate and used for the colorimetric assay of phenolic and polyphenolic compounds. It produces a blue color with molecules containing phenolic group. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent.

Structure of FC reagent in solution:

One hexavalent phosphomolybdotungstic acid complexes with the following structures formed in the solution.

$3\ H_2O \cdot P_2O_5 \cdot 13WO_3 \cdot 5MoO_3 \cdot 10\ H_2O$ and

$3\ H_2O \cdot P_2O_5 \cdot 14WO_3 \cdot 4MoO_3 \cdot 10\ H_2O$.

Basic mechanism of FC reagent:

When the organic compound reacts with hetero polyacid reagent under alkaline conditions undergoes reduction and is converted to molybdenum blue, which may be having either blue (or) green color with a λ_{max} of 620–780 nm.

The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotungstates- molybdates. Sequences of reversible one or two electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

Mo (VI) + e ⟶ Mo (V)

The wavelength of maximum absorption and stability and reproducibility of the reaction is dependent on pH, composition of hetero polyacid complex, the nature and concentration of reducing agent, temperature and time.

The drug (organic) probable effect a reduction of 1, 2 or 3 oxygen atoms from the tungstate and/or molybdate in FC reagent, thereby producing one or more feasible reduced species, which has the characteristic of intense blue color.

The reagent therefore measures the total reducing capacity of a sample, not just the level of phenolic compounds. The reagent has also been shown to be reactive towards thiols, many vitamins, the nucleotide base guanine, the trioses, glyceraldehyde and dihydroxyacetone, and some inorganic ions. Copper complexation increases the reactivity of phenols towards this reagent.

Factors effecting wavelength, stability and reproducibility of reaction:

The following are the five crucial factors which have to be taken into consideration during reaction. They are as follows:

- pH
- Composition of hetero poly acid complex
- Nature and concentration of a reducing agent
- Temperature
- Time

Preparation of FC reagent:

Into a 1500 ml flask introduce 25 gm of sodium tungstate, 25 gm sodium molybdate and 700 ml of water. To this, 50 ml of phosphoric acid and 100 ml of HCL are added. Reflux the mixture gently for about 10 hours and then 150 gm of lithium sulphate, 50 ml mixture without the condenser for about 15 min (or) until the excess bromine is expelled, cool & dilute with water. Before use, concentrated reagent is diluted with one part of filtrate with two parts of water.

Composition of reagent:

Sodium tungstate P	- 10 gm
Sodium molybdate	- 2.5 gm
Concentrated HCl	- 10 ml
Lithium sulfate	- 15 gm

General procedure for the estimation of drugs using FC reagent:

Drug solution is mixed with the alkali such as NaOH (phenolic 4% w/v) or Na_2CO_3 (amines 20% w/v). The FC reagent solution is added and keeps a side of the blue or green color production based on the nature of the compound. The colored solution is measured at 620 –780 nm.

Solution of the drug + alkali Solution + FC reagent ⟶ Intense blue color.

Storage:

FC reagent should be stored tightly capped at room temperature. Shelf-life of this reagent is greater than two years. If the solution acquires a greenish tint, it should be discarded. This stock solution was stored at a temperature not exceeding 4 °C.

Usage of the reagent:

- Folin & Ciocalteu's phenol reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically.
- It can also be used as a spray reagent in chromatographic procedures.
- The color development is due to the transfer of electrons at basic PH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence.
- The most common usage of this reagent is in the lowry method for determining protein concentration.

In this method, protein is pretreated with Copper (II) in a modified biuret reagent (alkaline Copper solution stabilized with Sodium potassium tartrate). The addition of Folin & Ciocalteu's phenol reagent generates chromogens that give an increasing absorbance between 550 nm and 750 nm. Normally, absorbance at the peak (750 nm) or shoulder (660 nm) is used to quantitate protein concentrations between 1-100 mg/mL while the absorbance at 550 nm is used to quantitate higher protein concentrations. In the absence of Copper, color intensity would be determined primarily by the tyrosine and tryptophan content of the protein, and to a lesser extent by cysteine, and histidine. Copper (II) enhances color formation by chelation with the peptide backbone, thus facilitating the transfer of electrons to the chromogens. Copper (II) has no effect on color formation by Tyrosine, Tryptophan or Histamine, but reduces that due to cysteine.

Applications of FC reagent:

- Used for the determination of adrenaline and non-adrenaline.
- Used for the determination of polyphenol content excreted in urine.
- Used for the determination of gatifloxacin based on the oxidative coupling reaction of reduced gatifloxacin with FC reagent in the presence of NaOH. The blue color solution is measured at 760 nm.
- Used for the determination of sample without $–NH_2$ and OH groups. *Examples:* Analgin, amidopine, acetazolamide, aspirin, acetanilide, barbituric acid, diazepam, etc.
- Used for the determination of drugs with NH_2 group. *Examples:* Thiamine HCl, *p*-amino benzoic acid, procaine HCl, ampicillin, thio acetazolamide, trimethoprim.
- Used for the determination of samples with $–NH_2$ and/or –OH groups. *Examples*: Folic acid, ascorbic acid, doxycycline, tetracycline, pyridine HCl, allopurinol, glucose, mannitol, sorbitol, etc.
- Used for the determination of samples with NH_2 and –COOH groups. *Example*: Tyrosine.
- Used for the determination of antibiotics. *Examples*: Azithromycin, roxythromycin, clarithromycin.

The method also was adapted to the analysis of aspirin-phenacetin-caffeine powders. A rapid and simple spectrophotometric procedure is described for the estimation of ajmaline and brucine. The method is based on the development of blue colored product due to reduction of tungstate and/or molybdate in Folin Ciocalteu's reagent (FCR) by ajmaline and brucine in alkaline medium. The color is stable for more than 48 hrs. The chromogenic reaction has λ max at 540 nm. In the alkaline medium, ajmaline and brucine react instantaneously with the FCR resulting in blue colored products.

Both the alkaloids seem to be due to a common reaction mechanism, i.e., the oxidation of the alkaloids and the reduction of FCR (blue color). A strict sequence of addition of reagent is necessary as it plays an important role. The blue color formation by FCR with alkaloids seems analogous to Folin phenol protein reaction.

Applications of FC reagent:

Folin-Ciocalteau reagent is extensively used chromogenic reagent for colorimetric estimation of different therapeutic agents. Table 6 shows the list of drugs, estimated by FC reagent.

Table 6: List of drugs estimated with F.C reagent.

Names of the drug react with F.C reagent
Acetanilide, acetazolamide, aspirin allopurinol, amidopyrine, analgin, ascorbic acid or vitamin C, barbituric acid and its derivatives, buspirone HCl, buspirone HCl, clozapine, clopidogrel, diazepam, diloxanide furoate, doxycycline, enrofloxacin, famotidine, folic acid, glucose, hetrazan, isoprenaline sulphate, isoxsuprine HCl, lomefloxacin, mannnitol, mebeverine HCl ¸ meloxicam, metoclopramide HCl, norfloxacin, orciprenaline sulphate, oxymetazoline HCl, para amino salicylic acid, phenacetin, pholedrine sulphate, piroxicam, pyridoxine HCl, salbutamol sulphate, salmeterol xinafoate, sildenafil citrate, sparfloxacin, sulphamethoxazole, terbutaline sulphate, tetracycline, thiamine or vitamin B_1, trimethoprim, nitrendipine nimesulide (after reduction).

1, 2 - Naphtha Quinone-4-Sulphonate Sodium (NQS) (Folin's reagent)

NQS [30-37] is the most commonly utilized chromomeric agent for the determination of a number of drugs containing 1° aromatic amine. It is an orthoquinone derivative, and generally useful for the colorimetric and visible spectrophotometric estimation of drugs containing either aliphatic or aromatic amino groups. Chemical structure of Folin's reagent is shown in fig 23.

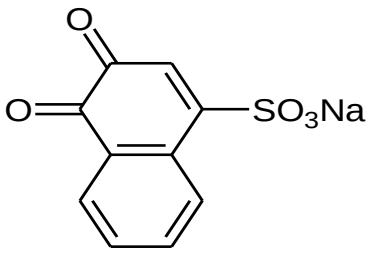


Figure 23. Chemical structure of Folin's reagent.

Mechanism of action

The mechanism of action of the NQS reagent involves when the NQS treated with the any amine-containing compound that will release the hydroxyl group and the sodium sulphonate group is replaced with the aromatic amine group. When the solution of the drug is allowed to react with NQS under alkaline conditions, a colored chromogen is formed. Fig. 24 shows the formation of colored chromogen with amines.

Procedure:

Mix a 50 ml portion of an aqueous amine solution with 10 ml of 0.138% sodium 1,2-naphthoquinone-4-sulphonate and 1 ml of pH 10.3 phosphate batter in 125 ml glass stoppered flask. Add 10 ml of chloroform to a teflon covered stirring box. Stopper the flask and vigorously stir the contents electro-magnetically for 20 minutes. The phases are separated, pipette out the chloroform layer and measure the absorbance at 450 nm against chloroform as blank.

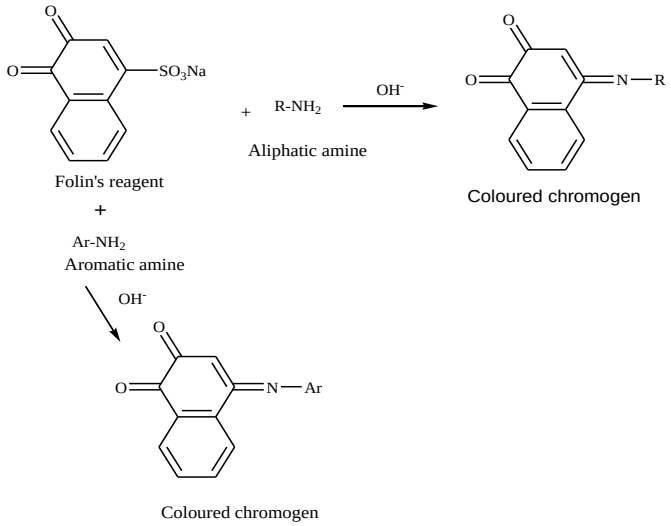


Figure 24: Formation of colored chromogen with amines.

Preparation of NQS reagent: Dissolve 260.20 g of 1, 2, naphtha quinine-4-sulphonate in 100 mL of water.

Applications:

Table 7. Drugs usually analyzed by NQS reagent.

S.NO.	Names of the drug react with Folin's reagent
1	Sulphadiazine, sulphadimidine, sulphamethoxazole, sulphisoxazole, sulphamerazine, sulphathiazole, sulphaguanidine, sparfloxacin, prochlorperazine, dapsone, ergot alkaloid, tranexamic acid, piroxicam, nateglinidine.
2	After reduction: Nimesulide, chloramphenicol, nimodipine, nitrendipine.
3	After hydrolysis: Sulfaacetamide sodium, succinyl sulfathiazole, phthalyl sulfathiazole.

Estimation of tranexamic acid:

Tranexamic acid is utilized for the treatment of haemophilic patients to stop haemorrhage. Drug solution is added to 0.5 ml NQS solution in a 10 mL volumetric flask. Heat the solution for 30 min in a boiling water bath. Cool the solution and make up the volume with distilled water. A reddish orange colored solution is obtained and it is measured at 474 nm. (fig 25).

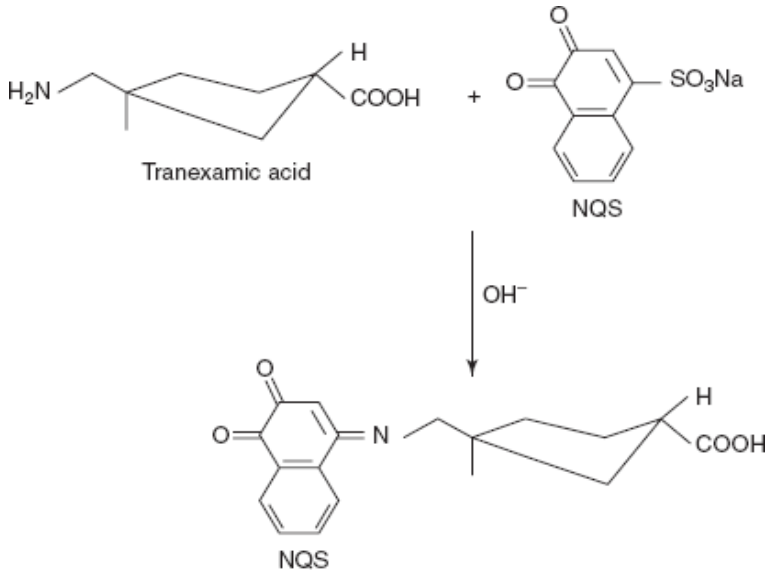
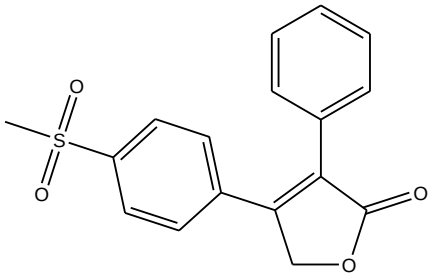


Figure 25: Formation of Reddish orange colored chromogen.

Determination of rofecoxib:

Rofecoxib is used as non-steroid anti-inflammatory drug chemically. It is 4-[4C methyl sulphonyl)-phenyl]-3-phenyl-2 (5, 4)-furanone.



Rofecoxib

Figure 26: Structure of Rofecoxib.

Mechanism of action of Rofecoxib

The lactone ring in Rofecoxib is changed to hydroxy acid by Na_2CO_3. NQS acts as an oxidising agent and abstracts hydrogen ions from hydroxy acid and forms probably the brown colored chromogen which is measured at 440 nm against the reagent blank.

2, 6-DICHLOROQUINONE-4-CHLOROIMIDE (GIBB'S REAGENT)

It is chiefly utilized for the identification and determination of phenols. It is very sensitive to phenols. Dacre first reviewed the Gibb's reagent [38-39]. Chemical structures of 2, 6-Dichloroquinone-4-Chloroimide and 2, 6- Dibromoquinone -4-Chloroimide is shown in fig 27 and fig 28 respectively.

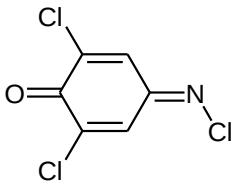


Figure 27. 2, 6 - Dichloroquinone -4-Chloroimide

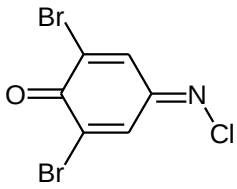


Figure 28. 2, 6 - Dibromoquinone- 4-Chloroimide

Principle and mechanism of action

When phenolic compounds react with Gibb's reagent gives imines. Gibb's reagent involves the solvolysis of the reagent which forms the 2, 6-dichloro quinone mono imines. This reacts with *para* substituted phenolic compounds and it forms the adduct which is formed by the attachment of the *para* position of the phenolic compound with the 2, 6-dichloro quinone mono imine. Then the adduct undergoes the deprotonation and forms the colored 2, 6-dichloro indophenols product which is measured at 500–670 nm (fig. 29).The reaction is independent of the pH of the medium. It also gives a positive reaction with *p*-alkoxy phenols, *p*-substituted aldehydes, *and p*-substituted halogen phenols. The pH of the medium is maintained at 9.24 with borate buffer.

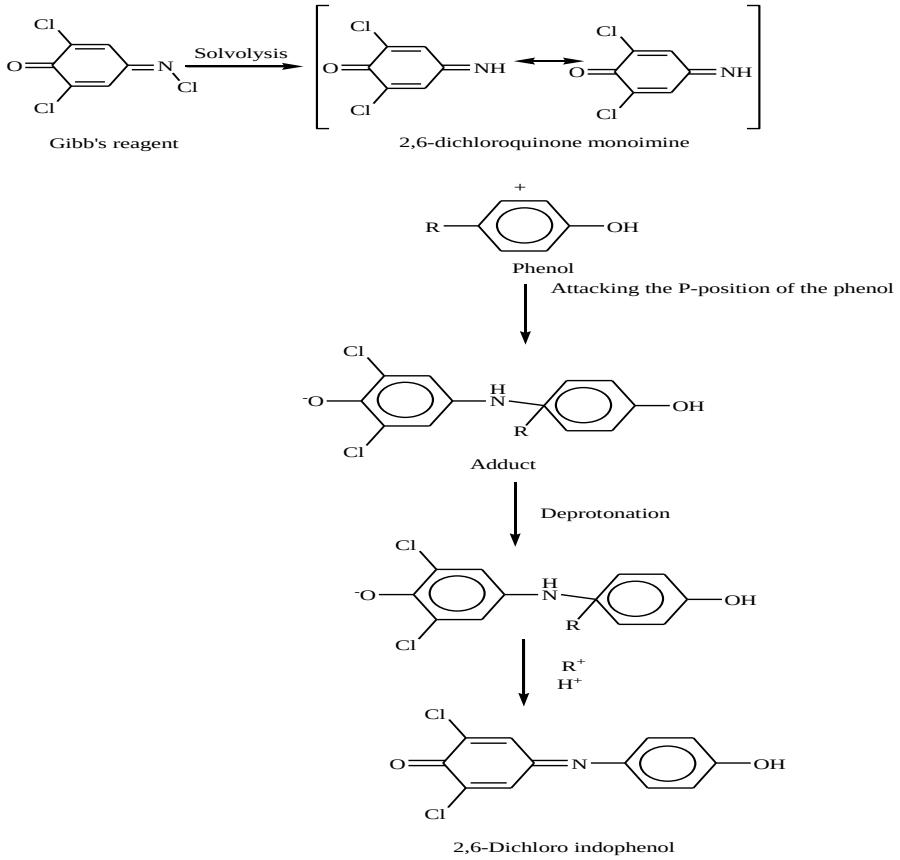


Figure 29. Mechanism of action of Gibb's reagent.

Applications: Determination of pyridoxine:

Sample solution is mixed with the Gibb's reagent and the solution pH are maintained at 9.24 by the addition of the NaOH. The blue colored solution is measured at 650 nm. (Fig. 30).

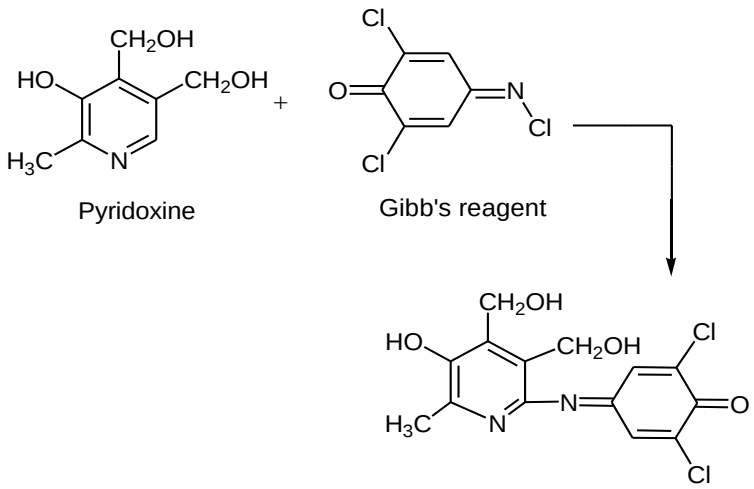


Figure 30. Formation of blue color indophenol.

Applications of Gibb's reagent:

- Used in the estimation of phenylephrine.
- *Used in the estimation of steroids Gibb's reagent*: Produces pink color with steroids, which is measured at 530 nm.
- *Used in the estimation of methoxalates*: Drug solution is heated with the NaOH to undergo the hydrolysis process. This hydrolysis produce the phenolic compound. Then this phenolic compound obtained is reacted with Gibb's reagent which produces a pink color which is measured at 530 nm.
- *Used in the estimation of nylidrine:* Drug solution is mixed with the Gibb's reagent and the borate buffer which produces the blue color and is measured at 610 nm.
- *Used in the estimation of amoxicillin:* Drug solution is mixed with Gibb's reagent and the borate buffer which produces the blue color and is measured at 610 nm.
- *Used in the estimation of rifampicin:* Drug solution is mixed with Gibb's reagent and the pH is maintained at 7.2 with weak alkali. The violet color solution is measured at 545 nm.

Table 8. List of drugs analyzed with Gibb's reagent.

The names of the drugs reacting with Gibb's reagent
Salicylamide, amoxycillin, piperazine, oxyphenbutazone, salbutamol, primaquine, methyl dopa, resorcinol, thymol, rifampcin, cloxacillin, piroxicam, barbiturates, guaiacol sulphate, stanazol, pyridoxine, anti epileptic drugs, thiamine, cimetidine, nylidrine, isoxsupurine, labetalol, aminophylline, famcyclovir, cefadroxil, captopril.

OXIDATION FOLLOWED BY COMPLEXATION

Examples: Bathophenanthroline, 1,10-phenanthroline, 2,2'-Bipyridine

Bathophenanthroline

Synonyms for bathophenanthroline are 4,7-Diphenyl-1,10-phenanthroline, BPhen. Empirical formula is $C_{24}H_{16}N_2$. Molecular weight of bathophenanthroline is 332.40. It is used in the estimation of many drugs. It is also used for the determination of iron in urine, serum, determination of Iron (II) in the presence of iron (III), for the determination of total iron. BPhen is a chelating agent and is particularly for ferrous ions. It was used in an ultra-sensitive and selective for a non extractive quenchofluorimetric method for determination of palladium (II) at µg/l levels. Bathophenanthroline is also being utilized as the buffer layer to improve the performance of organic photovoltaic cells.

It is extensively used chromogenic reagent used for the colorimetric or visible spectrophotometric determination of a number of drugs and pharmaceutical drugs. Chemically, it is a 4, 7-diphenyl-1, 10-phenanthroline. It is also known as iron (II) reagent. Chemical structure of bathophenanthroline is shown in fig 31.

Examples of iron (II) reagents:

i) 1,10-phenathroline or O-phenanthroline.
ii) 2,2'-Bipyridyl or 2,2'-Bipyridine.
iii) 2,2':3',2''- terpyridine (α,α′,α″-Tripyridyl, 2,6-Di(2-pyridyl)pyridine).
iv) O-Nitrosoresorcinal monomethyl ether (NRME).
v) 2,4,6-Tripyridyl-s-triazine (TPTZ).

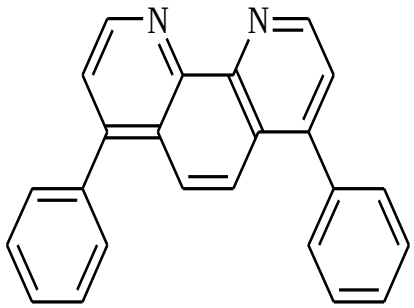


Figure 31: Structure of 4, 7-diphenyl-1, 10-phenanthroline.

Mechanism:

The analysis of drugs by using bathophenanthroline [(40)] involves two steps. Initially the solution of the drug under investigation is allowed to react with bathophenanthroline and ferric chloride (at elevated temperature). During this process, the drug gets oxidized and results in the generation of ferrous iron (II) [i.e., ferric chloride is converted to ferrous chloride]. The ferrous now complexes with bathophenanthroline to form a red colored choromogen. In this method, the reason of the addition of phosphoric acid is to stop the photochemical reduction. The following are the chemical reactions involved:

i) Drug + $FeCl_3$ ⟶ oxidized form of drug + Fe (II).

ii) 3 Bathophenanthroline + Fe (II) ⟶ Red colored complex (535 nm) which is shown in fig 32.

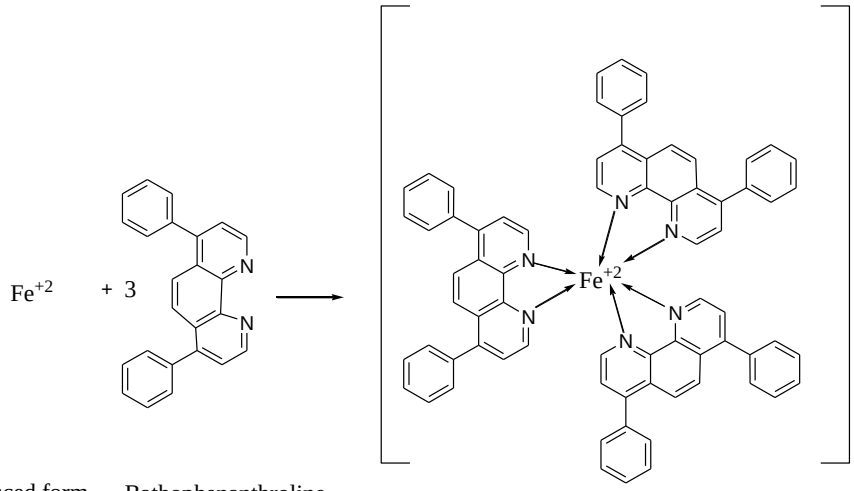


Figure 32. The formation of complex with bathophenanthroline forms red colored chromogen.

Ferric salts play a prominent role in the colorimetric estimation of organic compounds. Various phenols, hydroximic acid esters and more complicated compounds containing the phenolic OH groups in their molecule react with ferric salt in an aqueous media to give the intense coloration characteristic of each particular phenol. The color is due to the powerfully ionized complex phenolates of tervalent iron, which is formed according to the equation.

$$FeCl_3 + ArOH \longrightarrow 6H^+ + 3\,Cl + [Fe\,(OAr)_6]^3$$

The color intensity and stability of the complexes enhances when the –COOH group is adjacent to phenolic hydroxyl (e.g., Salicylic acid). The addition of acid, glycerol, alcohol, and sometimes excess ferric chloride decreases the degree of phenolate dissociation (hence the concentration of color decreases), and the color of the solution vanishes. Alkalizing also destroys the color by binding the iron ion into hydroxide.

The amides and esters of fatty acids are characterized by oximes with which the NHOH group is substituted for the NH_2 group. The substitution takes place during boiling with a solution of hydroxylamine salts.

$$R\,COO\,C_2H_5 + NH_2OH \longrightarrow R\,CO\,NH\,OH + C_2H_2OH$$
$$R\,CO\,NH_2 + NH_2OH \longrightarrow NH_3 + R\,CO\,NHOH$$

Hydroximic acids that are formed in the reaction can easily detect since they react with an ion of tervalent iron to give intensely colored complex salts. The nitro group is strong electron acceptor produces a clear – I effect (Inductive) in an organic molecule. Nitro methane is a pseudo acid, and displays tautomerism.

$$CH_3\,NO_2 \longrightarrow CH_2 = NOOH\ (isonitro\ group)$$

The sodium salt of nitro methane reacts with $FeCl_3$ to give a complex iron salt, which are intensely colored complex salts. The nitro group compounds. $FeCl_3$ reacts with sodium acetate to give first ferric acetate, which is straightaway hydrolyzed to give a complex compound, chlorides of use ferric hexa acetate (brown color)

$[Fe\,(OH)_3(COO)_6]^+\,Cl^-$

Poly hydroxy alcohols (or) oxy acids react with $FeCl_3$ to give stable complexes. Oxy acids react with $FeCl_3$ to give stable complexes. Oxy acids react with $FeCl_3$ to give complex salts of iron, which at the same time oxidize the oxy acids.

Ferric chlorides can also oxidize phenols. It oxidizes hydroquinones to quinones, which then gives quinhydrones. Napthols are oxidized by $FeCl_3$ to give sparingly soluble dinapthols, in which two napthelene rings are combined.

$$3C_{10}H_7OH + 2FeCl_3 \longrightarrow HOC_{10}H_6C_{10}H_6OH + 2\,FeCl_2 + 2HCI$$

Other phenols also form phenolates of iron with partial oxidation.

Acting as an oxidant a ferric salt is converted into ferrous salt. They can easily be detected by the usual reagent for divalent iron, potassium ferricyanide, 1, 10 phenanthroline, bipyridyl, triazine, or bathophenanthroline:

$$Fe2^{+} + [Fe(CN)_6]3^{-} \longrightarrow Fe_3[Fe(CN)_6]_2$$

1, 10 Phenanthroline forms a complex of low absorbence value with Fe (III) which in turn functions as a superior oxidant than Fe (II) itself. The reduction product is tries complex of Fe (II), well known as ferroin. Based on its complexing tendency and oxidizing properties, ferric salts were suggested in the estimation of several drugs.

2.2'- Bipyridine forms a complex of low tinctorial value with Fe (III) which in turn functions as a better oxidant than Fe (II) itself. The reduction product is tries complex of Fe (II). Based on its complexing tendency and oxidizing properties, ferric salts were suggested in the determination of several drugs.

Procedure for analysis:

Drug solution was added with bathophenanthroline and ferric chloride reagent and heated in a boiling water bath for 30 minutes and cooled to room temperature and add it with O-phosphoric acid solution and volume was filled up to (10 ml or 25 ml) level and the red colored chromogen was measured at 535 nm.

The intention of addition of O-phosphoric acid is to avoid photo chemical reduction of ferric chloride.

Applications:

Table 9. Drugs analyzed by bathophenanthroline.

Names of drug react with Bathophenanthroline
Salbutamol sulphate, terbutaline sulphate, isoxsuprine HCl, pholedrine sulphate, nylidrine HCl, salmeterol xinofoate, vitamin E analogues, vitamin A, nitrendipine.

1, 10-Phenanthroline

Synonym for 1,10-phenanthroline is o-phenanthroline. Its molecular weight is 180.21. Empirical formula is $C_{12}H_8N_2$. It is extensively utilized chromogenic reagent used for the colorimetric or visible spectrophotometric determination of a number of drugs and pharmaceutical substances. It is also known as ortho- phenanthroline (*O*- phenanthroline) [41-44]. It is constantly utilized in combination with a popular oxidant or oxidising agent namely ferric chloride. The chemical structure of 1, 10 phenanthroline is shown in fig. 33.

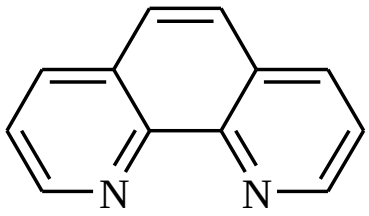


Figure 33. Structure of 1, 10-phenanthroline.

Mechanism

The analysis of drug (for example Prulifloxacin) by using 1,10- phenanthroline involves two steps. Initially the solution of the drug under investigation is allowed to react with 1,10- phenanthroline and Ferric chloride (at elevated temperature). During this process, the drug gets oxidized and results in the generation of ferrous iron [i.e., ferric chloride is converted to ferrous chloride, i.e., (II)]. The ferrous now complexes with 1,10- phenanthroline to form an orange red colored complex (fig 34). In this method, the reason of the addition of phosphoric acid is to stop the photochemical reduction. The following are the chemical reactions involved:

i) Drug + $FeCl_3$ $\longrightarrow$ Oxidised form of drug + Fe (II).

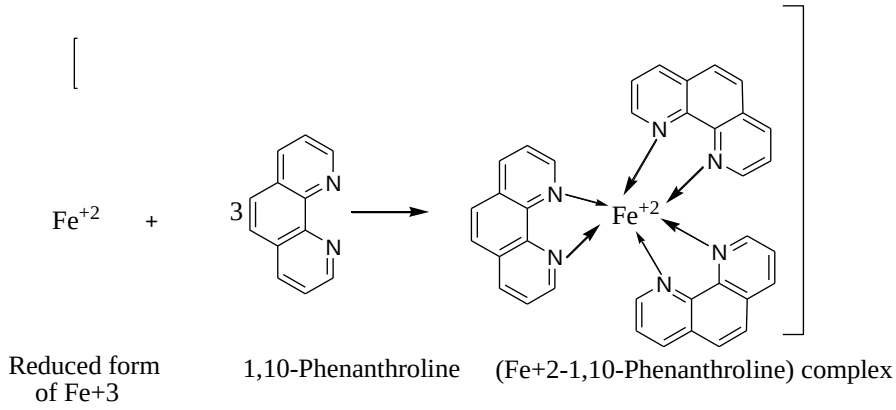


Figure 34. Formation of complex with 1,10-phenanthroline forms orange red colored chromogen.

Preparation of reagents:

Aqueous ferric chloride (0.4% w/v):

0.4 gm of ferric chloride was weighed accurately and dissolved in distilled water in 100 mL volumetric flask. The volume was brought up to the mark with distilled water.

1,10 - Phenanthroline:

(0.2% w/v in alcohol): precisely weighed 0.2 gm of 1,10 - phenanthroline mixed with distilled alcohol in 100 ml volumetric flask. The volume was made up to the mark with distilled water.

Procedure for analysis:

Drug solution was mixed with 1,10 - phenanthroline and ferric chloride reagent and heated in a boiling water bath for 30 min and cooled to room temperature and mix it with O-phosphoric acid solution and volume was made to a fixed (10 ml or 25 ml) level and the orange red colored chromogen was measured in between 500-520 nm. Purpose of addition of O-phosphoric acid is to prevent photo chemical reduction of ferric chloride.

Applications

Table 10. Drugs analyzed by *O*-phenanthroline.

Name of drugs determined by O-Phenanthroline
Salbutamol sulphate, terbataline sulphate, isoxsuprine hcl, nylidrine hcl, pholedrine sulphate, salmeterol xinafoate, tocopherols (or) vitamin e analogues, vitamin a, nitrindipine, nimeslide, oxymetazoline hcl, xylometazoline hcl, cefatoxame sodium, cefpirome sulphate, analgin, indomethacin, atorvastin, rosuvastatin, prulifloxacin, amisulpride.

2,2'-Bipyridine (Emmerie-Engel's reagent), or 2,2'- dipyridine or α,α'- bipyridine.

Emmerie-Engel were first used in the iron- 2,2'- bipyridine reaction for the determination of tocopherols. It is also known as Emmerie-Engel's reagent or 2, 2'- bipyridyl [45-47]. 2,2'- Bipyridine is used chromogenic reagent employed for the quantitative determination of several drugs or pharmaceutical substances by colorimetric or visible spectrophotometry. A structure of 2, 2' - bipyridyl is shown in fig 35.

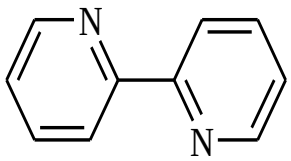
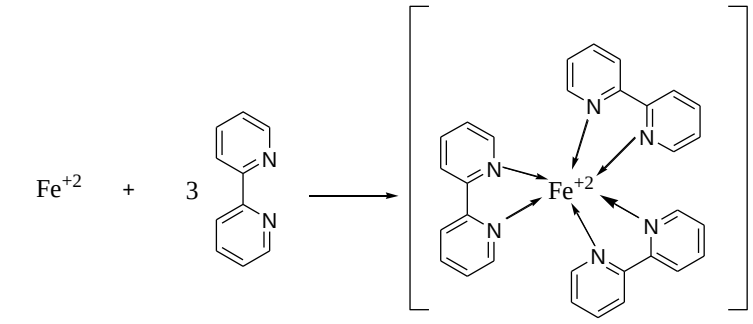


Figure 35. Chemical structure of 2, 2'-Bipyridyl.

Mechanism:

The analysis of drugs by utilizing 2,2'- Bipyridine is in two steps. At first the solution of the drug is allowed to react with ferric chloride. During this process, the drug gets oxidized and results in the generation of ferrous iron [i.e., ferric chloride i.e., Fe^{+3} is converted to ferrous chloride, i.e., Fe^{+2}]. In the second step the ferrous complexes with 2,2'- Bipyridine to form a orange colored complex which is shown in fig 36. In this method, the purpose of addition of phosphoric acid is to prevent the photochemical reduction. The chemical reactions involved is shown below:

i) Drug + $FeCl_3$ ⟶ Oxidised form of drug + Fe (II).



Reduced form of Fe^{+3} | 2,2'- Bipyridyl | (Fe^{+2}-2,2'- Bipyridyl) orange coloured complex

Figure 36. Formation of orange complex with 2,2' - Bipyridyl.

Preparation of reagents:

Aqueous ferric chloride (0.4 % w/v):

0.4 gm of ferric chloride was weighed accurately and dissolved in distilled water in 100 mL volumetric flask. The volume was brought up to the mark with distilled water.

2,2'-Bipyridine:

(0.2 % w/v in alcohol): An accurately weighed 0.2 gm of 2,2'-Bipyridine dissolved in distilled alcohol in 100 ml calibrated flask. The volume was filled up to the mark with distilled water.

Procedure:

Same as 1,10- phenanthroline.

Applications:

Table 11: Drugs analyzed by 2, 2'Bipyridyl Reagent.

S.No	Drugs Analyzed
1	Salbutamol sulphate, terbutaline sulphate, nylidrine HCl, pheoledrine sulphate, oxymetazoline sulphate.
2	Nephazoline sulphate, xylometazdine HCl, nimesulide (after reduction), adrenaline, nimodipine (after reduction), noradrenaline.
3	IsoxsuprineHCl, salmeterol xinafoate, ambroxyl HCl, lomefloxacin HCl, clopidogrel.

METOL (Oxidation followed by charge transfer)

Metol (P-N-methyl aminophenol sulphate) [48-51] is a bifunctional substrate. Chemical structure of Metol is shown in fig 37.

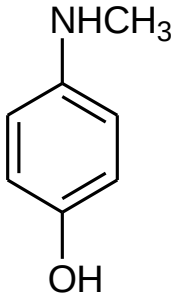


Figure 37. Chemical structure of metol.

Mechanism:-

a. Formation of a charge-transfer (CT) molecular complex.
b. Oxidative coupling.
c. Formation of ternary complexes.

When metol is oxidised, the following reaction takes place which is shown in fig 38.

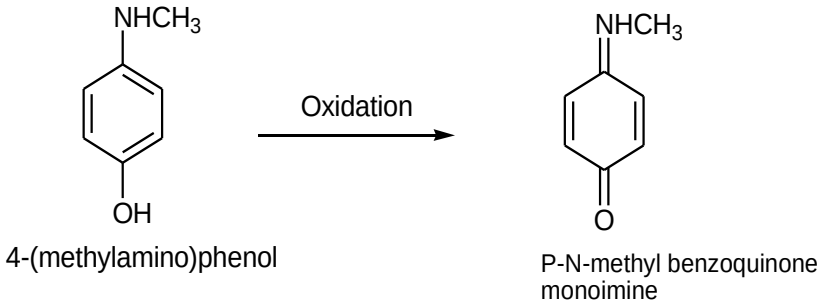


Figure 38. Formation of PMBQMI.

Formation of a charge-transfer (CT) molecular complex:

CT formed between an electron donor and electron acceptor. Some quinones are chloranil, fluoranil, N-aryl sulphonyl p-benzoquinone monoamine are used as acceptors.

Metol in the presence of NBS (N-bromosuccinimide) forms PNBQMI (P-N- methyl benzoquinone monoimine and later reported to form CT complex with primary amines (R-NH_2). This reaction is particularly used for the spectrophotometric determination of sulpha drugs and primary arylamines.

Metal is a bifunctional substrate, when treated with an oxidizing agent, undergo oxidation with 2 electron transfer to form very unstable and highly reactive PNBQMI. The reaction of the drug molecule with the PNBQMI formed in situ from metol and oxidizing agent result in the formation of a color product. In Metol -sulphanilamide system formation of colorful species was formed via a charge transfer molecular complex. The formation of charge-transfer complex, involving two moles of PNBQMI and one mole of sulphanilamide may be represented as electron transfer and hydrogen bond formation between quinoneimino oxygen and amino hydrogen of sulfanilamide. Fig 39 shows the formation of charge transfer molecular complex.

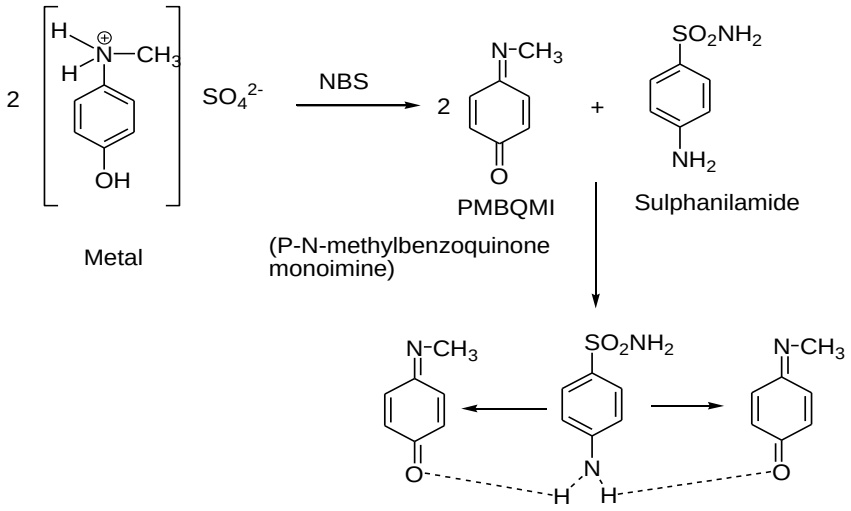


Figure 39. Formation of charge transfer molecular complex.

Oxidative coupling:

Quinones or quinone imines react with drugs containing imino, phenolic or thiol groupings. Metol may undergo an oxidative reaction with drugs containing these groupings to yield the substitution product (fig 40).

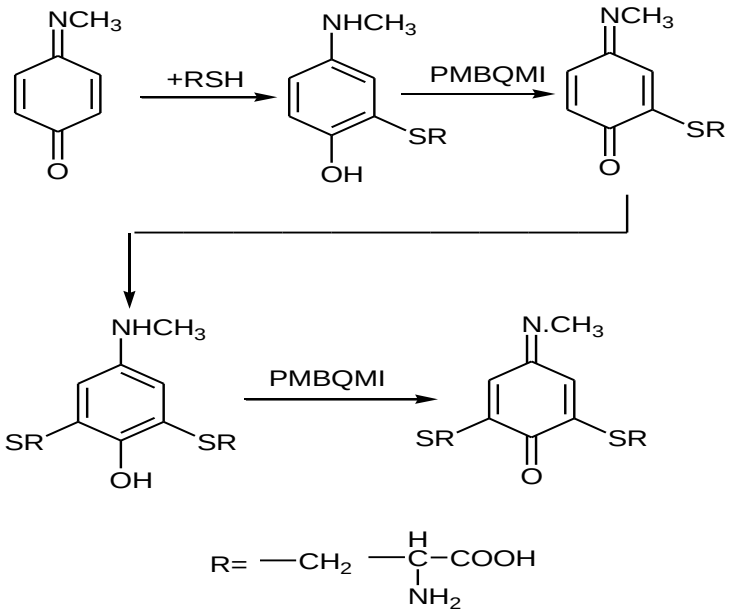


Figure 40. Oxidative coupling.

Formation of ternary complexes:-

Oxidation of metol with oxidants like hydrogen peroxide is very slow. Presence of metal ion enhances the process. Assume that three components, metol, metal ion, and hydrogen peroxide yield a ternary complex at pH 3.0 which decomposed and give us PMBQMI and free catalysing ion. The second product is then formed in the reaction between PMBQMI in the protonated form and the free metol still present to form a colored species which are stabilised in the form of an adduct with hydrogen peroxide.

Procedures employed in the determination of various kinds of compounds with METOL:

Direct spectrophotometric methods:

METOL is converted to PMBQMI by an oxidant which is reacting with the drug molecule with a colored species with a characteristic wavelength. The absorbance is directly praportnal to the concentration of the drug molecule. Most of the metol determinations belonging to direct spectrophotometric methods.

Differential spectroscopic methods:

Direct determination is not possible this method is adopted. Color developed by formation of CT complex between PMBQMI and sulphanilamide. Then drug is added to the colored species so formed and decrease in intensity of the colored species is measured. Drug concentration is directly praportnal to the decrease in intensity of the colored species.

Indirect spectrophotometric methods:

This method is used in some cases in which the drug molecule is highly susceptible the action of oxidising agent is then determined using metol-sulphonamide reagent. The consumed oxidising agent corresponds to the drug concentration.

Abbreviations:

BM reagent	= Bratton–Marshall reagent (BM reagent);
NED	= *N*-1-naphthyl ethylene diamine dihydrochloride;
MBTH	= 3-Methyl- 2- benzothiazolinone hydrazone hydrochloride;
PDAB	= Para dimethyl amino benzaldehyde;
PDAC	= para dimethyl amino cinnamaldhyde;
FC reagent	= Folin – Ciocalteu reagent;
NQS	= 1, 2-Naphthoquinone-4-sulfonate sodium;
BPhen	= Bathophenanthroline;
PNBQMI	= P-N- methyl benzoquinone monoimine.

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Conflict of interests:

Declare none.

CONCLUSION

Based on the functional group present in a molecule, the Bratton-Marshal (BM reagent), 3-Methyl-2-Benzothiazolinone Hydrazone Hydrochloride (MBTH reagent), para dimethyl amino benzaldehyde (PDAB reagent), Para dimethyl amino cinnamaldehye (PDAC reagent), Folin-Ciocalteu (FC reagent), 1, 2-Naphtha Quinone-4-Sulphonate Sodium (NQS reagent), 2,6-Dichloroquinone-4-Chloroimide **(**Gibbs reagent), Bathophenanthroline, 1,10 phenanthroline, 2,2'-Bipyridine, Metol reagents have been most usually employed for quantification of the drug molecule. We are confident that most of the chromogenic reagents, reaction mechanisms and method of analysis described in the above review article shall give the most stimulating learning experience and will enable you to understand the excitement of this interesting field. Since novel molecules are consistently chemical modifications of existing drug substance, this compilation should be helped to the analysts in developing appropriate methods of analysis for latest drug molecules and also be aware of the chemistry involved and the confidence to tackle novel situations. We do hope our attempt in this direction will aid students to perform practicals with better understanding. Over the years it was observed that at the postgraduate level numerous practicals in pharmaceutical analysis were based on the methods described in this review article. We expect this review article on "review of reagents used in spectrophotometry" should make drug analysis by spectrophotometry easier and better understand for analysts, educators and students.

REFERENCES

1) Ravisankar panchumarthy, Lokapavani CH et al. Novel sensitive spectrophotometric methods for determination of retigabine in bulk and pharmaceutical formulations. *International Journal of Pharma Sciences*. 2014; 4 (6): 773-779.
2) Nagamalleswari G, Phaneemdra D, et.al. New colorimetric method development and validation of sulfacetamide in bulk and formulation by different analytical reagents. *International Journal of Advances in Pharmaceutical Sciences*. 2013; 3(2): 30-36.
3) Panchumarthy Ravisankar et.al. Five novel spectrophotometric methods for quantitative determination of Prulifloxacin in pure and pharmaceutical formulations. *Asian Journal of Biomedical and Pharmaceutical Sciences*. 2015; 5(48): 01-13.
4) Sushma K, Somsubhra Ghosh, et.al. Role of chemical and analytical reagents in colorimetric estimation of pharmaceutical. *International Journal of Medicine and Pharmaceutical Research*. 2013; 1(5): 433-445.
5) Rahul Reddy M B, Gurupadayya B M et.al. Spectrophotometric determination of Lamuvidine using acidic dye and coupling reagent. *Indian Journal of Chemical Technology*. 2011; 18: 431-438.
6) Sri Varsha M, Raghavendra Babu N et.al. Development of new spectrophotometric method for estimation of tenofovir disoproxil fumarate using MBTH reagent. *International Current Pharmaceutical Journal*. 2015; 4(4): 378-381.
7) Padmalatha H, Anupama V, et.al. Spectrophotometric estimation of chlorthalidone in pharmaceutical formulation using MBTH & FC reagent. *World journal of Pharmacy And Pharmaceutical Sciences*. 2014; 3(2): 1443 -1440.
8) Sheeja Velayudhan Kutty, Susamma Cicy Eapen, et.al. Validated UV-Visible spectrophotometric method for the estimation of fenofibrate in pure and pharmaceutical formulation using MBTH reagent. *International Journal of Pharmaceutical Sciences and Drug Research*. 2012; 4(1): 74-76.
9) Shravya A, Chandan R.S, et.al. Spectrophotometric determination of Ezetimibe using MBTH reagent in pharmaceutical dosage form. *International Journal of Research in Ayurveda & Pharmacy*. 2011; 2(2): 521-525.
10) Ravisankar P, Lokapavani Ch, Devadasu Ch. Development of a validated spectrophotometric methods for the quantitative estimation of retigabine in bulk and pharmaceutical formulations. Indo American Journal of Pharm Research 2014:4(09).
11) Revanasiddappa H.D, Manju B. Spectrophotometric determination of antidepressant drugs using 3-methylbenzothiazoline-2-one hydrazone. *European Journal of Pharmaceutical Sciences.* 1999; 9(2): 221-225.
12) Sreedhar K, Sastry CS, et.al. Spectrophotometric methods for the determination of prazosin hydrochloride in tablets. *Talanta*. 1996; 43(11): 1847-55.
13) Hauser T.R. and Cummins R.L. Increasing sensitivity of 3-Methyl-2-Benzothiazolone hydrozone test for analysis of aliphatic aldehydes in air. *Anal. Chem*. 1964; 36(3): 679-68.
14) Sawicki E, Stanley T.W, Hauser T.R, Elbert W and Nee J.L. Spot test detection and colorimetric determination of aromatic amines and imino heteroaromatic compounds with 3-Methyl-2-benzothiazolone Hydrazone. *Anal.Chem*. 1961; 33(6): 722-725.
15) Dipak D. Patil, et.al. Spectrophotometric method for pregabalin determination: an experimental design approach for method development. *Journal of the Association of Arab Universities for Basic and Applied Sciences*. 2016; 21: 31-37.
16) Siddappa K, et.al. Spectrophotometric Quantitative determination of ambroxol hydrochloride in bulk and pharmaceutical dosage forms using PDAB Reagent. *International Journal of Pharmaceutical Sciences And Research*. 2015; 1(11): 4188-4194.
17) Patel KM, Patel CN, Panigrahi B et.al. Development and validation of spectrophotometric methods for the estimation of mesalamine in tablet dosage forms. *J Young Pharm*. 2010; 2(3): 284-288
18) KAPSE G. K., PRABHAKAR G., et.al, spectrophotometric methods for the estimation of nitazoxanide in pharmaceutical formulation. *Indian J. Pharm. Sci.,* 68 (3), 2006, 403-406.

19) Prakash S. Sarsambil, Gowrisankar D et.al. Visible spectrophotometric determination of ganciclovir by condensation and oxidative coupling reactions. *International Journal of ChemTech Research*. 2010; 2(1): 282-285.
20) Saifulla Khan P, Raveendra Reddy P et.al. Visible spectrophotometric methods for the determination of zolmitriptan in bulk and pharmaceutical formulations using aromatic aldehydes and Folin's reagent. *International Journal of ChemTech Research*. 2013; 5(6): 2941-2946.
21) Sivakumar R, Nallasivan, et.al. Visible spectrophotometric estimation of diacerein in bulk and pharmaceutical dosage forms. *Journal of Young Pharmacists*. 2010; 2(4): 414-416.
22) Viplava Prasad1 U, Syam Bab M, et.al. Development of new visible spectrophotometric methods for quantitative determination of almotriptan malate as an active pharmaceutical ingredient in formulations. *International Journal of Drug Development & Research.* 2012; 4(2): 369-374.
23) Anjoo Kamboj, Ritika Gupta, Ankita Rana et.al. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from extracts of Terminalia Bellerrica. *European Journal of Biomedical and Pharmaceutical Sciences.* 2015; 2(3): 201-215.
24) singhvi et.al. Visible Spectrophotometric estimation of aceclofenac and indapamide from tablets using Folin -Ciocalteu reagent. *Indian journal of pharmaceutical Sciences*. 2007; 69(1): 164-165.
25) Vinay Kumar N, Patibandla, Anusha P, et.al. UV- visible spectrophotometric estimation of olmesartan in pharmaceutical dosage form. *J Uma rao Research Desk*. 2016; 5(1): 573-578.
26) Shantaram Nangude, et.al. Development of UV spectrophotometric method for estimation of clarithromycin in pharmaceutical dosage forms by using Folin-Ciocalteu Reagent. *International Journal of Pharmaceutical and Chemical Sciences.* 2013; 2(2): 640-642.
27) Mounika CH., Umadevi N, et.al. New visible spectrophotometric methods for the estimation of silodosin in pharmaceutical formulations. *International Journal Of Research In Pharmacy and Chemistry.* 2013; 3(3): 595-597.
28) Kanakapura B. Vinay, et.al. Sensitive spectrophotometric determination of tramadol hydrochloride in pharmaceuticals using Folin- Ciocalteu's Reagent. *Turk j Pharm*. 2013; 10(1): 57-68.
29) Roopa Kothathi Papanna, Jayanna Bidapur Krishna Gowda, et.al. Redox reaction based spectrophotometric assay of some drugs in pharmaceuticals. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2015; 7(8): 151-156.
30) Ibrahim A. Darwish, Heba H. Abdine, et.al. Simple spectrophotometric method for determination of paroxetine in tablets using 1,2-Naphthoquinone-4-Sulphonate as a chromogenic reagent. *International Journal of Analytical Chemistry*. 2009; 1-8.
31) Xu L, Wang H, Xiao Y. Spectrophotometric determination of ampicillin sodium in pharmaceutical products using sodium 1,2-naphthoquinone-4-sulfonic as the chromogentic reagent. *Spectro chimica Acta Part A*. 2004; 60(13): 3007–3012.
32) Li Q-M, Li J, Yang Z-J. Study of the sensitization of tetradecyl benzyl dimethyl ammonium chloride for spectrophotometric determination of dopamine hydrochloride using sodium 1, 2-naphthoquinone-4-sulfonate as the chemical derivative chromogenic reagent. *Analytica Chimica Acta*. 2007; 583(1): 147–152.
33) Panda S.S, Ravikumar B.V.V, et.al. Spectrophotometric determination of alendronate sodium by using Sodium- 1, 2-Napthaquinone -4-sulphonate. *International Journal of Pharmaceutical Sciences and Nano- technology.* 2012; 4(2): 1563-1568.
34) Biksham Babu A, Ramu G, et.al. Spectrophotometric determination of an Antiretroviral Drugs Stavudine in Bulk and Pharmaceutical Formulations. *Rasayan J. Chem*. 2011; 4(2): 336-342.
35) Abdalla Ahmed Elbashir and Salwa Fwad Awad. A new spectrophotometric method for determination of penicillamine in pharmaceutical formulation using 1, 2-naphthoquine-4-sulfonate (NQS). *J Pharmacovigilance.* 2013; 1(2): 1-5.
36) Kobayashi Y. Kubo H. and Kinoshita T. Fluorometric determination of guanidino compounds by new post column derivatization system using reversed-phase ion-pair high-performance liquid chromatography. *Annal. Biochem*. 1987; 160(2): 392-398.
37) Darwish A. Kinetic spectrophotometric methods for determination of trimetazidine dihydrochloride. *Analytica Chimica Acta.* 2005; 551 (2): 222-231.
38) Mohamad Nasir Mat Arip. Reaction of 2, 6 dichloroquinone-4-chlorimide (gibbs reagent) with permethrin- an optical sensor for rapid detection of permethrin in treated wood. *Chemistry Central Journal*. 2013; 7: 122-124.
39) Suobodovao, Krenek P, Fraenkl M, Gasparic J, the color reaction of phenols with the gibbs reagent- the properties of the colored product and the optimum reaction conditions, *Microchimica Acta*, 11, 1978, 197-211.
40) Nasrin Banu Shaikh Ismail, & Badiadka Narayana. Spectrophotometric methods for the determination of anti-diabetic drug glipizide in pure and pharmaceutical formulations. *Eurasian Journal of Analytical Chemistry.* 2017; 2(1): 83-93.
41) B M Gurupadayya, et.al. Spectrophotometric determination of meloxicam by Sodium nitroprusside and 1, 10 phenanthroline reagents in bulk and its pharmaceutical formulation. *Indian Journal of Chemical technology.* 2013; 20: 111-115.
42) Abdellaziz LM, Hosny MM. Development and validation of spectrophotometric atomic absorption and kinetic methods for the determination of Moxifloxacin hydrochloride. *Analytical Chemistry Insights*. 2011; 6: 667-78.
43) Ravisankar P, Devadasu Ch et.al. New spectrophotometric methods for the quantitative analysis of Prulifloxacin in pharmaceutical dosage forms. *Int. J. Chem. Sci*. 2010; 8(4): 2309-2324.
44) Panchumarthy Ravisankar. Five novel spectrophotometric methods for quantitative determination of Prulifloxacin in pure and pharmaceutical formulations. *Asian Journal of Biomedical and Pharmaceutical Sciences.* 2015; 5(48): 01-13.
45) Ayesha Syeda, Mahesh H.R.K, Akheel Ahmed Syed. 2,2' Bipyridine as a new and sensitive spectrophotometric reagent for the determination of nanoamounts of certain dibenzazepine class of tricyclic antidepressant drugs. *Farmaco.* 2005; 60 (1): 47-51.
46) Neeha Tadipaneni and Devala Rao Garikapati. New spectrophotometric methods for the determination of Entacapone in bulk and pharmaceutical dosage forms. *Elixir Appl. Chem*. 2011; 38: 4064-4065.

47) Jyothirmayee D, Sudhakar Sai Babu G, et.al. Spectrophotometric determination of Gemifloxacin in pharmaceutical formulations. *Asian Journal of Chemistry*. 2010; 22(2): 1634-1636.
48) Kishore Medikondu, Kalyani Ch. S.R.G. Development of spectrophotometric methods for quantitative determination of 7-ADCA in pharmaceutical formulations. *Asian Journal of Research in Chemistry*. 2011; 4(2): 272-277.
49) Mohan Krishna, Jayachandra reddy P. Spectrophotometric methods for the assay of Ceftazidime in bulk and its pharmaceutical formulations. *Chem Sci Trans*. 2013; 2(2): 684-690.
50) Seshagiri V.M., Mohan Rao Tadi, Reddy R.S. Spectrophotometric methods for the determination of Nelfinavir Mesylate, *Journal of Analytical Chemistry*, 2004; 59(6): 552–556.
51) Basavaiah K. Spectrophotometric determination of zidovudine in pharmaceuticals based on Charge-Transfer complexation involving N-Bromosuccinimide. Metol and sulphanilic acid as reagents. *E-Journal of Chemistry*, 2007; 4(2): 173-179.