

Five novel spectrophotometric methods for quantitative determination of Prulifloxacin in pure and pharmaceutical formulations

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ABSTRACT :

The main objective of this present work is to successfully develop one UV and four visible new spectrophotometric techniques. One UV and four visible spectrophotometric methods have been developed depending on the reactivity of various structural units for instance piperazine ring and tertiary amine and carbonyl group in Prulifloxacin. It is observed that the statistical analysis results of correlation coefficient of each method values are observed to be greater than 0.999 which speaks that the proposed methods have good linearity. The order of sensitivity of the said proposed methods are $M_1 > M_2 > M_5c > M_5b > M_5a > M_4 > M_3$. The % RSD values are observed below two for all methods which shows that all the proposed methods are precise. The results of analysis of the pharmaceutical formulations assert that the proposed methods are safely be adopted for routine quality control of Prulifloxacin in bulk and pharmaceutical preparations to yield correct and accurate analysis results.

Keywords: Prulifloxacin, Visible spectrophotometric methods, Bratton-Marshall reagent, Validation.

INTRODUCTION:

Prulifloxacin (PRFX) pertains to prodrug of ulifloxacin which is broad spectrum fluoroquinolone an anti-bacterial agent. Prulifloxacin is metabolized in the body to the active compound in Ulifloxacin. Prulifloxacin is a prodrug and found to be effective on par with Ciprofloxacin, co-amoxiclav or Pefloxacin in the treatment of bronchitis exacerbations and lower urinary tract infections. PRFX is not official drug in IP, BP and USP pharmacopoeia. In this connection relevant numerous literature survey of PRFX was compiled and thoroughly examined before development of these methods to have adequate knowledge in this regard. Literature survey disclosed that very few spectrophotometric methods have been reported so far. Majority of HPLC methods were applied in determination of FQs in human plasma¹⁻⁵, edible animal products, feeds and to a lesser extent in pharmaceutical formulations⁶⁻¹⁰. Capillary Zone Electrophoresis¹¹ methods are in existence to determine the active metabolite of Prulifloxacin existed

in human plasma and some other biological fluids. It was also reported that there was a sensitive determination of Prulifloxacin owing to its fluorescence enhancement on Terbium (III)-Sodium Dodecylbenzene Sulfonate system¹². There are no analytical reports available to estimate PRFX for utilizing visible spectrophotometry. Therefore the author inclined to select PRFX for the development of sensitive, precise and accurate spectrophotometric methods validated according to ICH Q2(R1) guidelines¹³, depending on several chemical reactions duly involving the analytically prominent functional groups existed in the structure. Thus the author chooses one ultraviolet and four visible spectrophotometric methods namely M_1 , M_2 , M_3 , M_4 , and M_{5a} , M_{5b} , M_{5c} for determination of PRFX in bulk samples and pharmaceutical formulations. The broad details of different spectrophotometric methods developed are shown in Table 1.

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Table 1. Spectrophotometric methods developed for the assay of PRFX in pharmaceutical formulations.

Method No	Reagent/ solvent	Chemical principle involved	λ_{max} (nm)	Beer's law limits ($\mu\text{g/ml}$)	Reference
M ₁	0.1N NaOH	Ultra-violet absorption	275	1 - 10	Present chapter
M ₂	Ce (IV)/MBTH	Oxidative coupling	580	1 - 5	Present chapter
M ₃	FC	Redox reaction	700	10 - 50	Present chapter
M ₄	BTB	Ion-association complex formation	420	5 - 25	Present chapter
M _{5a}	Fe(III)/PTL	Oxidation followed by complex formation	520	5 - 30	Present chapter
M _{5b}	Fe(III)/BPN	Oxidation followed by complex formation	520	4 - 30	Present chapter
M _{5c}	Fe(III)/BPTL	Oxidation followed by complex formation	600	2 - 10	Present chapter

The author has developed a simple and sensitive UV method (water as solvent) and taken it as a reference method to compare accuracy of the results achieved through the proposed methods. The procedure for the assay of PRFX in pharmaceutical formulations is explained bellow.

Reference method for PRFX

Precisely weighed the powder of the tablets equivalent to 50 mg of the drug and it was dissolved in 50 ml of water duly shaken well and filtered. The resultant filtrate was diluted to 100 ml with water. Further, out of the diluted solution 5 ml of this solution was again diluted to 100 ml with water. Then the absorbance of the solution was determined at λ_{max} 275 nm (Fig. 1). The drug quantity was calculated from the Beer's law plot (Fig. 2) of the standard drug in water.

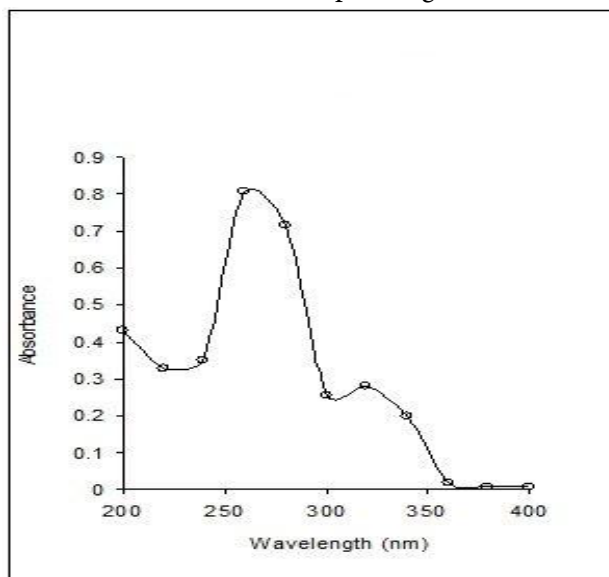


Fig. 1: Absorption spectrum of PRFX.

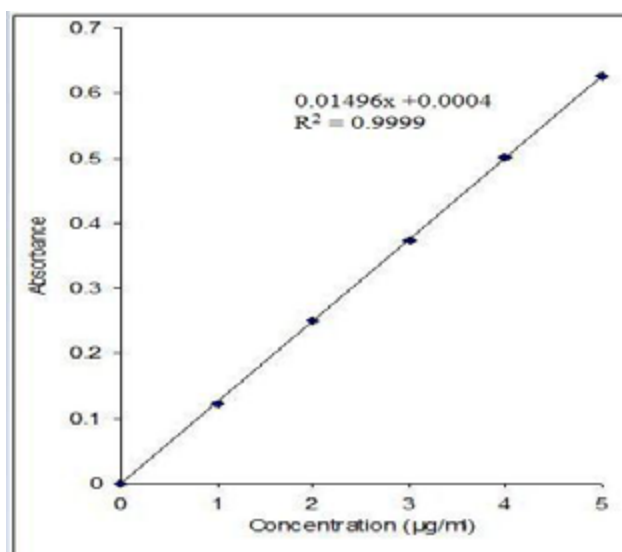


Fig. 2: Calibration curve for PRFX.

MATERIALS AND METHODS

Materials

All the chemicals and reagents used in the present study were of Anal R grade. The pure drug of Prulifloxacin was supplied by Hetero drugs Ltd., Hyderabad, A.P, India as a sample gift. The commercial formulation such as Unidrox and Pruquil were obtained from local market. Reagents like MBTH and bromothymol blue of Fluka company, Ceric ammonium sulphate ferric chloride and sodium hydroxide chemical of Merck company, Folin-Ciocalteu reagent of Loba company, 1,10 phenanthroline and 2,2'-bipyridyl of Qualigens company, Bathophenanthroline and orthophosphoric acid relating to CDH company were procured locally.

Instruments used

Systronics Double beam UV-Visible spectrophotometer 2201 with 1 cm correctly matched quartz cells was utilized for all spectral and absorbance measurements. A Systronics digital pH meter was employed for all pH measurements.

Preparation of Reagents and standards

Analytical grade chemicals and reagents were utilized and the solutions were made in triple double distilled water.

For method M_1

NaOH solution (0.1N)

Sodium hydroxide 0.1N solution was prepared by dissolving 4 grams of sodium hydroxide in 1000 ml of distilled water.

Method M_2

3-Methyl-2-Benzothiazolinone hydrazone solution

MBTH solution was prepared with 200 mg of 3-methyl-2-benzothiazolinone hydrazone aptly weighed duly dissolved in 100 ml of distilled water and got 0.2 % of MBTH (Fluka, 0.2 % w/v, 1.12×10^{-2} M).

Ceric ammonium sulphate (CAS)

Ceric ammonium sulphate was prepared by dissolving 500 mg of ceric ammonium sulphate in 100 ml of dilute sulfuric acid and got 0.5 % w/v CAS solution. (Merck, 0.1 % w/v, 6.17×10^{-3} M).

Method M_3

Folin-Ciocalteu reagent

FC reagent solution was prepared with Folin-Ciocalteu reagent (2 N) duly diluting three times with distilled water. (Loba, 0.67 N).

Sodium hydroxide solution

NaOH was made by dissolving 5.0 g of sodium hydroxide duly diluting with 100 ml of distilled water and got 5 N of NaOH (Merck, 5 % w/v, 1.89×10^{-1} M).

Method M_4

Bromothymol blue

Bromothymol blue was prepared with 200 mg of bromothymol blue accurately weighed duly dissolving in 100 ml of distilled water (washed cleanly with chloroform to get rid of any chloroform soluble impurities.) and got 0.2 % of bromothymol blue. (Fluka: 0.2 % w/v, 3.16×10^{-3} M).

Glycine buffer pH 2

Glycine buffer pH 2 was prepared by adding 50 ml of

glycine solution (0.1 M) with 50 ml of HCl (0.1 M) solution duly adjusted to pH 2.0.

For methods M_{5a} , M_{5b} , and M_{5c}

1, 10-phenanthroline solution (PTL) (M_{5a})

PTL solution was prepared with 198 mg of 1,10 phenanthroline weighed duly dissolving in 100 ml of 0.1N hydrochloric acid and eventually got 0.198 % of PTL. (Qualigens, 0.198 % w/v, 1.0×10^{-2} M).

2, 2'-bipyridyl solution (BPN) (M_{5b})

BPN solution was prepared with 156 mg of 2, 2'-bipyridyl precisely weighed duly dissolving in 100 ml of 0.1N hydrochloric acid and got 0.156 % of BPN. (Qualigens, 0.156 % w/v, 1.0×10^{-2} M).

Bathophenanthroline solution (BPTL) (M_{5c})

BPTL solution was prepared with 332 mg of Bathophenanthroline solution correctly weighed duly dissolving in 100 ml of ethanol and eventually got 0.332 % of BPTL. (CDH, 0.332 % w/v, 1.0×10^{-2} M).

Ferric chloride stock solution

$FeCl_3$ stock solution was prepared with 162 mg of anhydrous ferric chloride accurately weighed and duly dissolved in 100 ml of distilled water and got 0.162 % w/v of $FeCl_3$ (Merck, 0.162 % w/v, 3.3×10^{-3} M). 33.3 ml of above prepared stock solution was further diluted to 100 ml with distilled water.

Orthophosphoric acid solution (OPA)

OPA solution was prepared with orthophosphoric acid duly diluting 1.3 ml in 100 ml of distilled water. (CDH, 2.0×10^{-1} M).

Preparation of standard drug solutions

Method M_1

Exactly 100 mg pure drug of PRFX was put into a 100 ml volumetric flask which consists of 20 ml of 0.1N sodium hydroxide and sonicated for 15 min. The volume was filled to the mark with 0.1N sodium hydroxide solution to get the stock solution (1mg/ml) and it was again diluted with same solvent and got working standard solution of 100 μ g/ml.

Methods M_2 , M_3 and M_4

100 mg pure drug of PRFX was accurately weighed and put into a 100 ml volumetric flask which consists of 20 ml of 0.1N hydrochloric acid and sonicated for 15 min. The volume was filled to the mark with hydrochloric acid solution to get the stock solution (1mg/ml) and it was again diluted with same solvent and got working standard solution of 100 μ g/ml.

Methods M_{5a} , M_{5b} and M_{5c}

Precisely 100 mg pure drug of PRFX was transferred into a 100 ml volumetric flask consists of 40 ml of acetonitrile and sonicated for about 15 min. The volume was filled upto the mark with acetonitrile to get the stock solution (1mg/ml). This solution was again diluted with the same solvent and got working standard solution of 100 μ g/ml.

Recommended procedures

For bulk samples

Method M₁

Standard drug aliquots of PRFX (0.1 - 0.5 ml, 100 µg/ml) solution in 0.1N sodium hydroxide were poured into a series of 10 ml volumetric flasks. The volumes were filled upto the mark with 0.1N sodium hydroxide and the absorbance of this solution was measured at 275 nm against solvent blank. The quantity of drug was calculated from calibration curve.

Method M₂

Standard drug aliquots of PRFX (0.5 - 2.5 ml, 100 µg/ml) solution in 0.1N hydrochloric acid were shifted into a series of 10 ml volumetric flasks. To this 1.5 ml of ceric ammonium sulphate (0.5 % w/v) and 2 ml of MBTH was added and mixed thoroughly and the solution was allowed to react in the ambient temperature for 20 min. The bluish-green colored chromogen so formed was determined at 580 nm against the reagent blank. The quantity of PRFX was calculated from its linearity plot.

Method M₃

Standard drug aliquots of PRFX (0.1 - 0.5 ml, 1000 µg/ml) solution in 0.1N hydrochloric acid were poured into a series of 10 ml volumetric flasks. To this 1ml of Folin-ciocaltau reagent as well as 2 ml of sodium hydroxide (5 % w/v) solution were added and shaken thoroughly and the reacted mixture was kept for 15 min. The blue colored chromogen so formed was estimated at 700 nm against the reagent blank. The quantity of PRFX was calculated from its calibration plot.

Method M₄

Standard drug aliquots of PRFX (0.5 to 2.5 ml, 100 µg/ml) solution in 0.1M HCl was transferred into a series of separating funnels of 125 ml and equalized with water and added 4ml of buffer pH 2.0 and 2 ml of bromothymol blue (0.2 % w/v) and then the solution was saturated with aqueous phase up to 10 ml. The reacted mixture was shaken for 5 min and kept it duly to settle. Then the drug-dye complex was extracted into 10ml of chloroform. The yellow colored so formed in organic layer was taken up for absorbance measurements and it was estimated at 420 nm against the reagent blank. The quantity of PRFX was calculated from its calibration plot.

Methods M_{5a}, M_{5b}, and M_{5c}:

Standard drug aliquots of Prulifloxacin solution (0.5 to 2.5 ml, 100 µg/ml for M_{5a}, 0.4 to 3.0 ml for M_{5b} and 0.2 to 1.0 ml, 100 µg/ml for M_{5c}) transferred in to a series of 10 ml of volumetric flasks and the volume to each flask was made to 3 ml with acetonitrile. Then 1.5 ml of Fe (III) solution, 2 ml of PTL or BPN or BPTL solutions were added to each volumetric flasks. The said solutions were heated on a boiling water bath for thirty min. The above said solutions then cooled to room temperature then 2 ml of o-phosphoric acid solutions was added and the total volume was brought to 10 ml with acetonitrile. The absorbance values of

coloured solutions were measured after five min at 210 nm for M_{5a}, 520 nm for M_{5b} and 600 nm for M_{5c} against the respective reagent blank. Ultimately the amount of the PRFX was calculated from the calibration plots.

Pharmaceutical formulations

Method M₁

Twenty tablets of PRFX were exactly weighed duly crushed to form smooth powder. An amount of said tablet powder equivalent to 50 mg of PRFX was correctly weighed and shifted into a 100 ml volumetric flask consisting of 50 ml of 0.1N NaOH. The above said solution was sonicated for 15 min to get it extracted well and filtered through a cotton wool and the filtrate was filled up to volume with 0.1N NaOH. Then it was exactly diluted with the same solvent. Then working sample solutions were made and the same procedure laid down under bulk samples was adopted.

Methods M₂, M₃ and M₄

Twenty tablets of PRFX were correctly weighed duly crushed to form smooth powder. An amount of said tablet powder equivalent to 50 mg of PRFX was exactly weighed and shifted into a 100 ml volumetric flask consisting of 50 ml of 0.1N HCl. The said solution was well sonicated for 15 min to get it extracted well and filtered through a cotton wool and the filtrate was filled upto volume with 0.1N HCl. After words it was precisely diluted with the same solvent and utilized for M₂, M₃ and M₄. Then working sample solutions were made and the same procedure laid down under bulk samples was adopted.

For methods M_{5a}, M_{5b}, and M_{5c}

Twenty tablets of PRFX were rightly weighed and powdered smoothly. Out of the said tablet powder of PRFX equivalent to 50 mg was correctly put into a 100 ml volumetric flask having 50 ml of acetonitrile. The said solution was thoroughly sonicated to extract the drug for 15 min and got filtered through a cotton wool and the filtrate was filled up to volume with acetonitrile. Lastly the solution was again diluted with acetonitrile and got the strength of 100 µg/ml and 1000 µg/ml solution. There after working sample solutions were prepared duly following the procedure prescribed under bulk samples.

RESULTS

Spectral Characteristics

To determine the optimum wavelength relating to maximum absorption (λ_{max}), the spectra were scanned in the wavelength region of 400 - 800 nm against corresponding reagent blank. For each method the reagent blank absorption spectrum was noted against solvent used in each method and the results were graphically shown in the Figures from 3 to 9. The absorption curves of colored species formed in every method denotes maximum characteristic absorption but the blank in each method showed low or no absorption in this region.

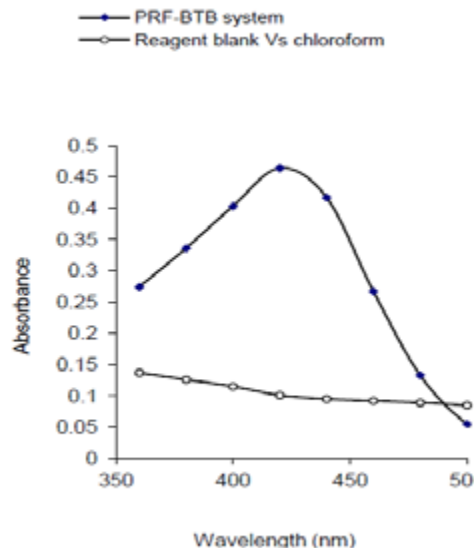
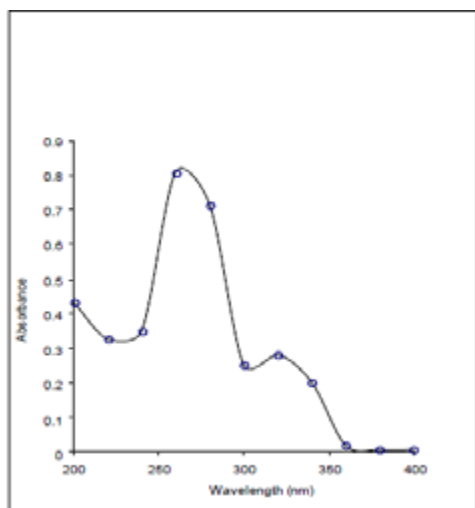


Fig. 3: Absorption spectra of PRFX with 0.1 N NaOH.

Fig. 6: Absorption spectra of PRFX with BTB system and its reagent blank.

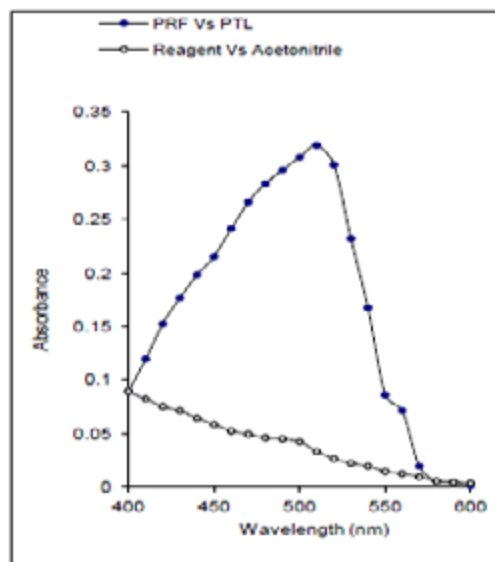
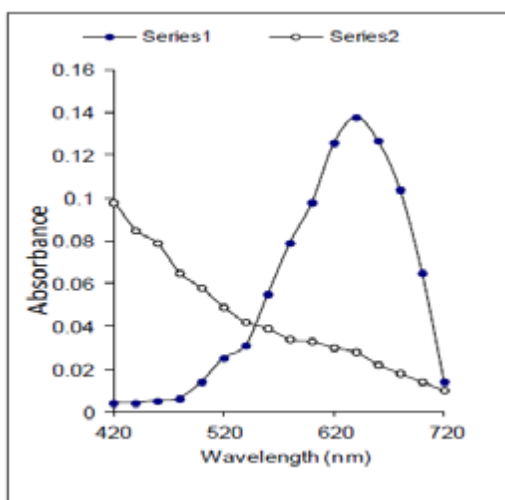


Fig. 4: Absorption spectra of PRFX with MBTH/Ce (IV) system and its reagent blank.

Fig. 7: Absorption spectra of PRFX with PTL system and its reagent blank.

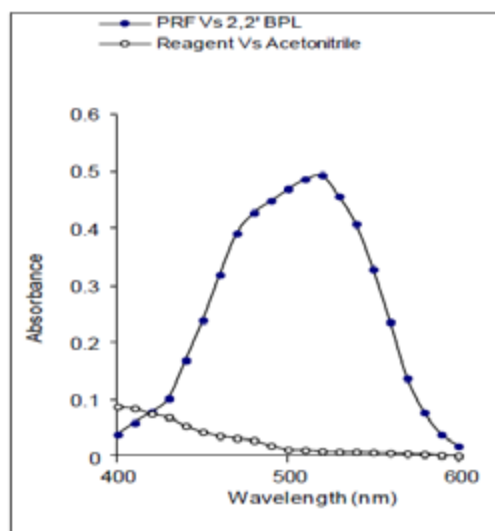
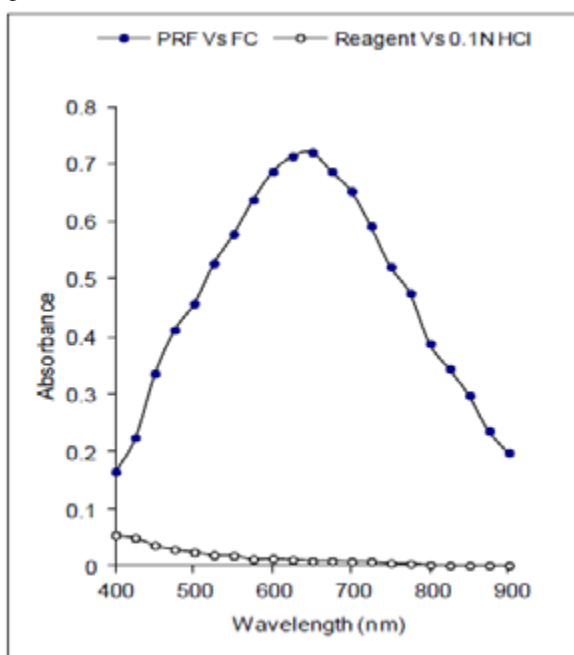


Fig. 5: Absorption spectra of PRFX with FC system and its reagent blank.

Fig. 8: Absorption spectra of PRFX with BPN system and its reagent blank.

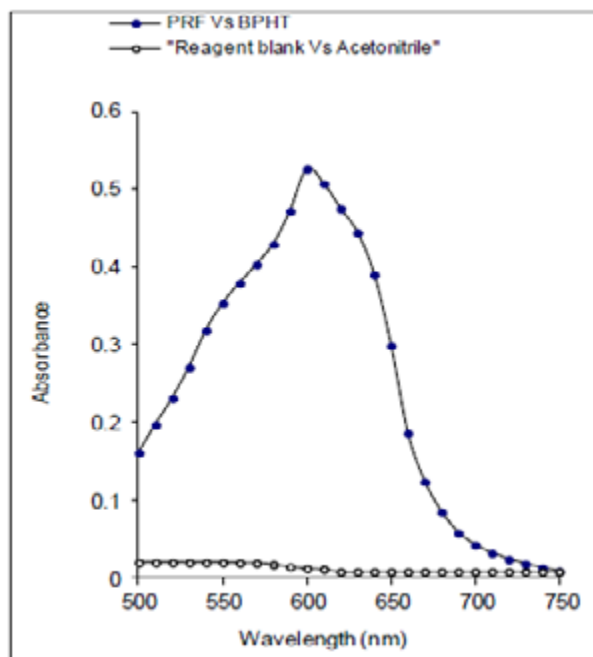


Fig. 9: Absorption spectra of PRFX with BPTL system and its reagent blank.

Optimum conditions for fixation of procedures

Fixation of parameters

While developing these methods, a detailed systematic study of the effects of numerous relevant parameters connected with methods were undertaken by changing one parameter at a time duly controlling all other parameters as they have to achieve optimum color development and least blank color, reproducibility and the reasonable period of stability of final colored species formed. The following studies on the methods (M_1 to M_3) were taken up for this purpose.

Method M_2

Depending on the reaction of PRFX with MBTH in presence of Ce (IV), this method was developed. The

optimum conditions in this method were fixed depending on the study of various relevant parameters that is the nature of oxidant, concentration of oxidant, concentration of MBTH, solvents utilized and stability period of colored species. The necessary optimum conditions keenly observed and results achieved were presented in Table 2.

Method M_3

This method involved the redox reaction between PRFX and Folin-Ciocalteu reagent. The effects of numerous parameters that is to say nature of alkali, reaction time, volume of FC reagent, volume of alkali, temperature and stability of colored species formed were carefully studied. The optimum conditions were observed and results achieved were presented in Table 3.

Method M_4

The best conditions for these method was fixed based on the detailed study of relevant parameters such as concentration of acid, concentration of dye bromothymol blue (BTB), choice of organic solvent, ratio of organic to aqueous phase, shaking time, temperature and stability of colored species in organic phase duly measuring the absorbance at appropriate λ_{max} 420 nm. The optimum conditions developed and real conditions selected for the procedure were shown in Table 4.

Methods M_{5a} , M_{5b} , and M_{5c} :

These methods are depending on the complete conversion of Fe (III) ions to Fe (II) ions by PRFX. The Fe (II) so produced gave a colored complex with PTL (M_{5a}) or BPN (M_{5b}) or BPTL (M_{5c}). The optimum conditions necessary for the formation of colored species were established by studying the effect of reagent concentration, Fe (III) concentration, heating time, solvent for eventual dilution. The results achieved were presented in Table 5.

Table 2: Optimum conditions established for method M2.

Parameter	Optimum range	Conditions in the procedure	Remarks
λ_{max}	570 - 590	620	----
Nature of oxidant	Ce (IV)	Ce (IV)	When the other oxidants like Fe (III), Cr (VI), CAT, IO_4^- , $S_2O_8^{2-}$, Fe (CN) $_6^{3-}$ utilized in lieu of Ce (IV) the absorbance of colored species were reduced.
Volume of MBTH, 1.12×10^{-2} M and keeping time	0.5 - 1.5 ml	1.0 ml	Over and above 1.5 ml, the absorbance of test solution stays on the same against the reagent blank.
Volume of Ce (IV)	1.5 - 2.5 ml	2 ml	If Ce (IV) volume increases more than 2.5 ml, the blank absorption increases.
Keeping time and temperature	10 - 25 min at room temperature.	15 min at room temperature	The organic solvents including ethanol and acetonitrile were tested. Methanol was selected for keeping the formed hydrazone product in solution form.
Order of addition of reagents	--	Drug, MBTH and Ce (IV)	The change in the order of addition of reagents results in the decrease in absorbance.
Solvent for final dilution	--	Distilled water	There is no change in the intensity of colored species produced even though water miscible organic solvents such as ethanol, propane-2-ol, or acetonitrile are utilized in place of water for final dilution.
Stability of colored species after final dilution	Immediate - 60 min	20 min	After 60 min the absorbance of colored species was observed to be slowly decreasing.

Table 3: Optimum conditions established for method M₃.

Parameter	Optimum range	Conditions in procedure	Remarks
λ_{\max} (nm)	650 - 750	700	----
Effect of volume of FC reagent	0.8 - 1.2 ml	1.0 ml	In excess of 1.2 ml of reagent shows blank interference.
Effect of volume of 5 % NaOH solution (ml)	0.8 - 1.2 ml	1.0 ml	Over and above 1.2 ml of NaOH solution shows low absorbance values.
Order of addition of reagents on color development	---	PRFX, FC, NaOH	Change of the order of addition of reagent resulted in decrease in absorbance.
Time for maximum color development (min)	2.0 - 4.0	3.0	Keeping for more than 4 min has no additional advantage.
Solvent for final dilution	---	Water	Among the different water miscible solvents tried. It was found that the water is the most excellent solvent.
Stability of the colored species (min)	Immediate to 60 min	10 min	The colored species were constant for 60 min. Then onwards the absorbance slowly decreases.

Table 4: Optimum conditions established for methods M₄.

Parameter	Optimum range	Conditions in the procedure	Remarks
λ_{\max} (nm)	410 - 430	420	-
Effect of pH	1.5 - 2.5	2.0	The extraction was feasible for estimation if the overall pH was maintained at 4.0.
Choice of organic solvent for extraction of the colored complex	---	Chloroform	The other water immiscible solvents tested for the extraction of coloured complex into organic phase include chlorobenzene, dichloromethane, carbon tetrachloride, benzene and n-butanol. Chloroform was chosen for its selective extraction of the coloured drug-dye complex from the aqueous phase.
Effect of the ratio of organic to aqueous phase on extraction	1:1.5	1:1	The extraction of colored species formed into chloroform layer was incomplete if the ratio of chloroform to aqueous phase was in excess of 1:2.
Effect of shaking time on extraction	1 - 5 min	3 min	Stable absorbance values were got at shaking period of 1-5 min.
Effect of temperature on the colored species (°C)	Lab. Temperature (28 °C ± 5)	Lab. Temp. (28 °C ± 5)	If the temperature is less than 20 °C the extraction of the colored species was found to be improper. At high temperatures (> 35 °C) the stability of the colored species was found to be less.
Stability of colored species	Immediate - 4 hours	10 min	The colored species in organic phase was stable up to 4.0 hours and then absorbance decreased slowly.

Table 5. Optimum conditions established for methods M_{5a}, M_{5b} and M_{5c}.

Parameter	Optimum range	Conditions in procedure	Remarks	
λ_{\max} (nm)			----	
For M _{5a}	515 - 525	520		
For M _{5b}	515 - 525	520		
For M _{5c}	595 - 605	600		
Effect of volume of 3.3 x 10 ⁻³ M Fe (III) solution for M _{5a} , M _{5b} .	0.8 - 1.2 ml.	1.0 ml.	Variation of the volume above and below this range gave inconsistent results.	
Effect of volume of 5.0 x 10 ⁻³ M Fe (III) solution for M _{5c} .	0.8 - 1.2 ml	1.0 ml		
Effect of volume of 1.0 x 10 ⁻² M PTL (M _{5a}), 1.0 x 10 ⁻² M BPN (M _{5b}), and 1.0 x 10 ⁻² M BPTL (M _{5c}).	0.8 - 1.2 ml	1.0 ml	Variation of the volume above and below these range exhibited irregular results.	
Effect of temperature on colored species	65 - 75 °C	70 °C	It was noticed that thermo stated boiling water bath was essential to maintain 70 °C uniformly for getting maximum color development. Below this temperature the strength of colored complex was less.	
Effect of heating time	For M _{5a} For M _{5b} For M _{5c}	NLT 30 min NLT 45 min NLT 10 min	35 min 50 min 15 min	Below the optimum conditions the colored complex was not produced.
Volume of 2.0 x 10 ⁻² M O - phosphoric acid required.		1 - 2 ml	2 ml	To complex excess of Fe (III) ions, a minimum of 1 ml of 2.0 x 10 ⁻² M o-phosphoric acid was necessary.

Table 5: (continued)

Parameter	Optimum range	Conditions in procedure	Remarks
Order of addition of reagents on color development			
For M _{5a}	-	PRFX, Fe (III) and PTL before heating and phosphoric acid after heating.	Due to interchange of the order of drug, Fe (III) and PTL or BPN or BPTL no effect was found on the absorbance.
For M _{5b}	-	PRFX, Fe (III) and BPN before heating and phosphoric acid after heating	
For M _{5c}	-	PRFX, Fe (III) and BPTL before heating and phosphoric acid after heating.	
Effect of temperature (°C)	70 - 100	80 °C for 15 min.	At room temperature no color was formed.
Nature of solvent for final dilution	-	Acetonitrile	It was observed that other solvents like methanol, acetone and dioxane did not increase the intensity of the eventual colored product.
Stability of colored species of the final dilution	Immediate 45 min	10 min	The absorbance of the colored product decreased gradually after 45 min.

Optical characteristics

In order to test whether the coloured species obtained in the proposed methods adhered to beer’s law, the absorbance at correct wavelength of a set of solutions containing different amounts of PRFX and specified amounts of reagents as given in indicated procedures for each method were recorded against the concerned reagent blanks. Optical characteristics, regression data, precision and accuracy of the proposed methods for PRFX are shown in the following Table 6. So as to check the validity of beer’s law for the said methods, the absorption at appropriate wavelengths of a set of solutions consisting of different quantities of PRFX and specified amounts of reagents as explained in the procedures for every proposed method were noted against concern reagent blanks and beer’s law plots of the systems were illustrated graphically (Figures 10 to16).

Table 6: Optical characteristics, regression data, precision and accuracy of the proposed methods for PRFX.

Parameter	M ₁	M ₂	M ₃	M ₄	M _{5a}	M _{5b}	M _{5c}
λ _{max} (nm)	275	580	700	420	510	520	600
Beer’s law limits (µg / ml)	1 - 10	1 - 5	10 - 50	5 - 30	5 - 25	4 - 30	2 - 10
Molar absorptivity (L. mole ⁻¹ cm ⁻¹)	3.941x 10 ⁴	1.347 x 10 ⁴	5.860 x 10 ²	9.459 x 10 ³	2.69x 10 ²	1.430x 10 ⁴	3.091x 10 ⁴
Detection limits (µg / ml)	0.1136	0.1131	0.9720	0.2720	0.105	0.101	0.0115
Sandell’s sensitivity (µg/cm ² /0.001 absorbance unit)	0.0066	0.03427	0.07874	0.0487	0.9615	0.040	0.0149
Optimum photometric range	1.5 - 4.5	2.5 - 11.5	8 - 44	5.5 - 29.5	4 - 24	8 - 24	4.5 - 19.5
Regression equation (Y = a+ bc): Slope (b)	0.1496	0.02951	0.01280	0.02054	0.00102	0.03099	0.0668
Standard deviation of slope (S _b)	6.64 x 10 ⁻⁵	6.62 x 10 ⁻⁵	1.24 x 10 ⁻³	1.11 x 10 ⁻³	1.003x 10 ⁻⁶	5.54x 10 ⁻⁵	3.86 x 10 ⁻⁵
Intercept (a)	0.004	0.00019	0.000952	-0.00095	-0.0010	0.00133	0.000523
Standard deviation of intercept (S _a)	2.58 x 10 ⁻³	1.003 x 10 ⁻²	3.773x 10 ⁻²	1.694 x 10 ⁻²	4.83x 10 ⁻⁴	9.51x 10 ⁻⁴	2.34 x 10 ⁻⁴
Standard error of estimation (S _e)	4.72 x 10 ⁻³	4.77 x 10 ⁻³	4.28 x 10 ⁻³	3.33 x 10 ⁻³	8.42x 10 ⁻⁴	4.28x 10 ⁻³	3.07x 10 ⁻³
Correlation coefficient (r)	0.9999	0.9999	0.9996	0.9998	0.9995	0.9999	0.9999
% Relative standard deviation*	0.681	0.3988	0.4121	0.3996	0.953	0.8492	0.2807
% Range of Error (Confidence limits)* 0.05 level 0.01 level	0.714 1.121	0.4185 0.6564	0.4325 0.6783	0.4194 0.6577	1.0003 1.568	0.8913 1.3978	0.2946 0.4620
% Error in bulk samples*	0.33	-0.51	0.25	-0.67	0.44	-0.19	0.75

* Average of six determinations.

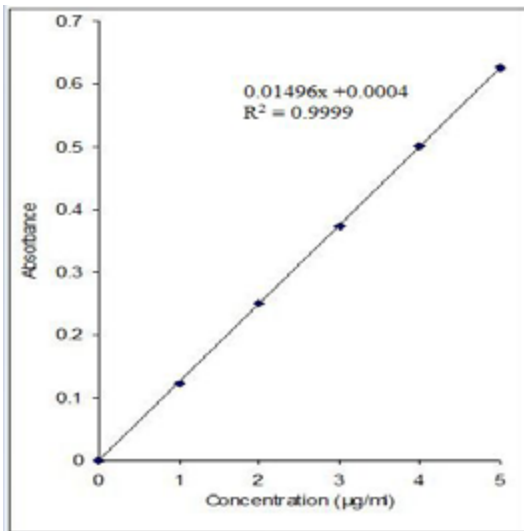


Fig. 10: Beer's law plot of PRFX with 0.1N NaOH system.

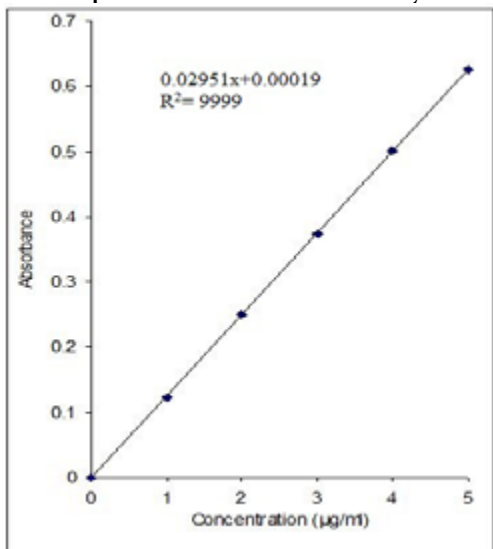


Fig. 11: Beer's law plot of PRFX with MBTH/Ce system.

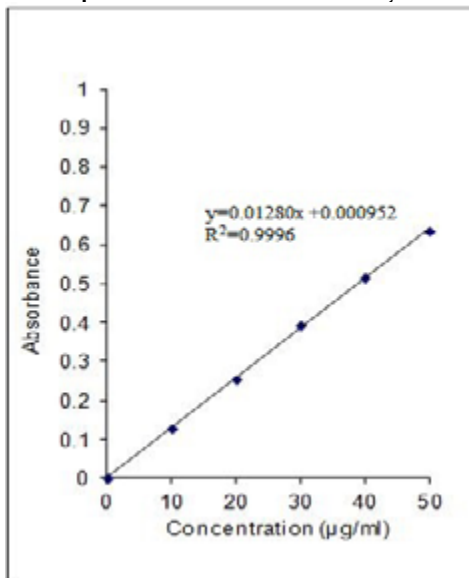


Fig. 12: Beer's law plot of PRFX with FC system.

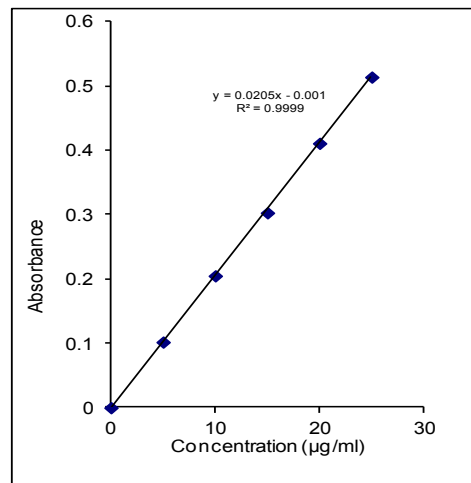


Fig. 13: Beer's law plot of PRFX with BTB system.

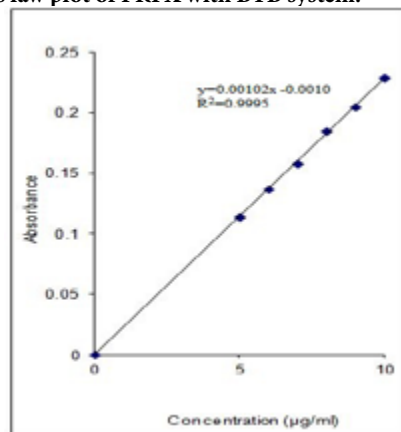


Fig. 14: Beer's law plot of PRFX with PTL system.

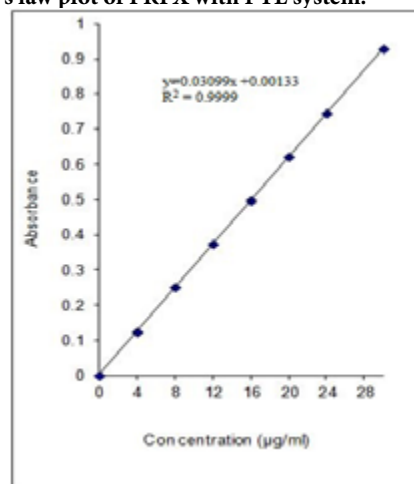


Fig. 15: Beer's law plot of PRFX with BPN system.

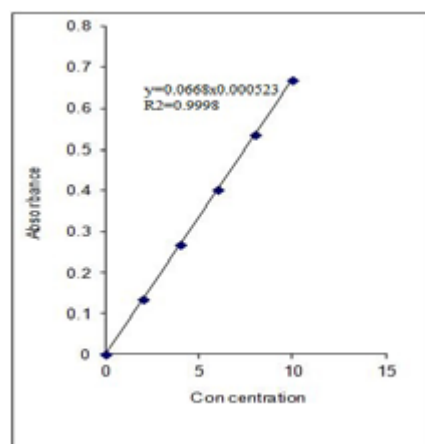


Fig. 16: Beer's law plot of PRFX with BPTL system.

Method validation

Precision

The precision of every one out of 4 proposed visible spectrophotometric methods and one UV were decided individually from the absorbances values got by actual estimation of 6 replicates of a fixed quantity of PRFX in 10 ml solutions for each replicate and the precision of the method was examined.

Accuracy of the method

By getting aliquots consisting of known quantities of bulk as well as pharmaceutical formulations of PRFX duly estimating them based on the reported and proposed methods, the accuracy of the method was decided. To explain the suitability of the proposed method the recovery studies of the PRFX were held.

Interference studies

The effect of broad range of excipients and other additives generally present in the formulations of PRFX were investigated separately and were determined under best conditions.

Table 7: Assay and recovery of PRFX in dosage forms.

Method	Sample	Labeled Amount (mg)	Proposed Method			Found by reference method \pm S.D	% Recovery by proposed methods** \pm S.D
			Amount found* (mg) \pm S.D	t (value)	F (Value)		
M ₁	T ₁	600	599.05 \pm 0.013	0.652	2.125	601.03 \pm 0.031	99.95 \pm 0.28
	T ₂	600	598.08 \pm 0.016	0.713	1.242	598.23 \pm 0.011	100.12 \pm 0.42
M ₂	T ₁	600	597.99 \pm 0.023	0.632	1.525	597.56 \pm 0.009	99.48 \pm 0.74
	T ₂	600	597.98 \pm 0.023	0.851	2.138	596.78 \pm 0.015	100.21 \pm 0.52
M ₃	T ₁	600	598.05 \pm 0.012	0.572	1.893	595.82 \pm 0.014	99.78 \pm 0.45
	T ₂	600	600.01 \pm 0.013	0.457	2.193	601.23 \pm 0.016	101.97 \pm 0.09
M ₄	T ₁	600	598.06 \pm 0.009	0.564	1.759	596.05 \pm 0.021	99.99 \pm 0.41
	T ₂	600	598.04 \pm 0.019	0.515	1.626	600.01 \pm 0.015	101.17 \pm 0.23
M _{5a}	T ₁	600	599.02 \pm 0.012	0.719	2.531	597.45 \pm 0.011	99.26 \pm 0.55
	T ₂	600	600.12 \pm 0.009	0.541	1.233	602.5 \pm 0.084	99.04 \pm 0.12
M _{5b}	T ₁	600	598.92 \pm 0.017	1.023	1.651	599.23 \pm 0.022	99.95 \pm 0.11
	T ₂	600	601.52 \pm 0.021	0.936	2.825	587.89 \pm 0.013	98.99 \pm 0.23
M _{5c}	T ₁	600	598.56 \pm 0.009	0.672	1.249	601.85 \pm 0.011	100.29 \pm 0.13
	T ₂	600	602.56 \pm 0.018	0.811	1.204	600.65 \pm 0.026	99.98 \pm 0.37

T₁ and T₂ are tablets of Unidrox and Pruquil

*Average \pm standard deviation of six determinations, the t and F- values refer to comparison of the proposed method with reference method. Theoretical values at 95 % confidence limits t = 2.571 and F = 5.05.

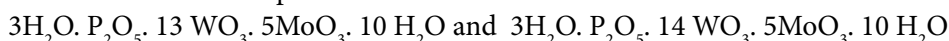
** Average of six determinations.

Chemistry of colored species

PRFX consists of different functional groups of several reactivity such as Tertiary amino group, piperazine and carbonyl functional groups. Five methods were developed in total depending on the reactivity of the said functional groups. The reaction involved in the above proposed methods showed several colored chromogens which are explicitly detailed by the scheme furnished here under scheme 1 to 4.

Method M₃

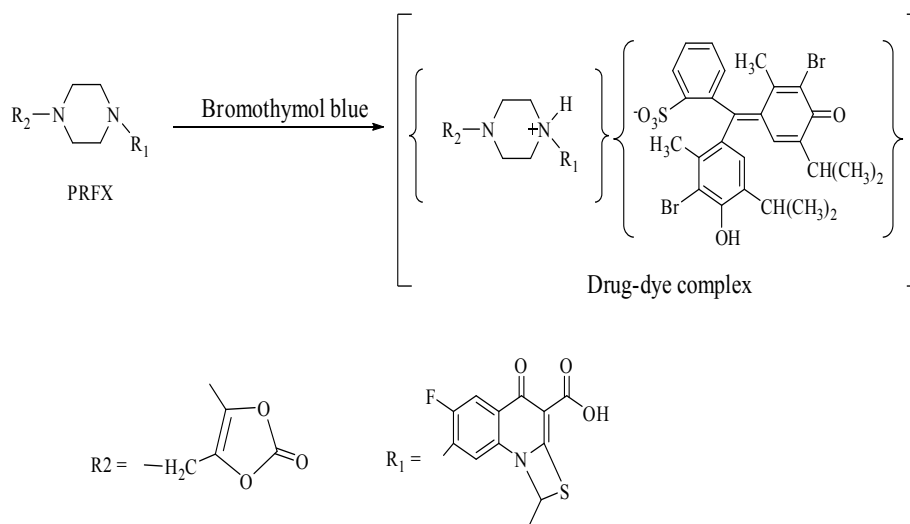
The color formation occurred by FC reagent with PRFX is explained as follows depending on the comparison with the reports of earlier inventors. Chemically FC reagent is phosphomolybdotungstic acid which is a heteropoly acid. It involves the chemical species as detailed bellow.



PRFX likely to effect reduction of 1, 2 or 3 oxygen atoms from the tungstate and/or molybdate, duly producing one or more feasible reduced species, which has characteristic of intense blue color.

Method M₄

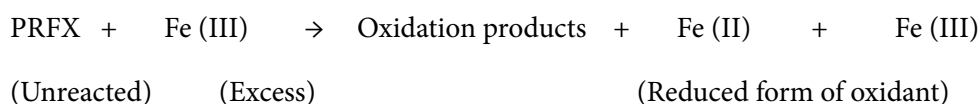
Because PRFX contains cyclic tertiary nitrogen, it produce ion association complex with acidic dyes BTB. It is extracted into chloroform from the aqueous phase. The protonated nitrogen with positive charge of PRFX molecule in acid medium is anticipated to attract the negative charge of the dye and act as a single unit by holding together with the help of electrostatic attraction. The study of slope ratio method showed that the ratio of drug to dye is 1:1. Considering the analogy of the structure with ion association complex is explained in scheme 1.



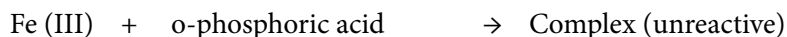
Scheme 1: PRFX reaction with bromothymol blue Methods M_{5a} , M_{5b} , and M_{5c}

PRFX shows reduced property owing to the existing of functional moieties (one or more) susceptible to oxidation selected oxidizing agents namely Fe (III) under controlled experimental conditions. When treated with known excess of oxidant, PRFX undergoes oxidation, yielding products of oxidation apart from reduced form of oxidant, Fe (II) from Fe (III), in addition unreacted oxidant. So there are feasible conditions to determine the drug content colorimetrically, which is equivalent to the reacted oxidant or reduced form. The reduced form of Fe III (Fe II) has a tendency to deliver colored complex when treated with 1, 10 - phenanthroline (M_{5a}), 2, 2'- bipyridyl (M_{5b}) and bathophenanthroline (M_{5c}).

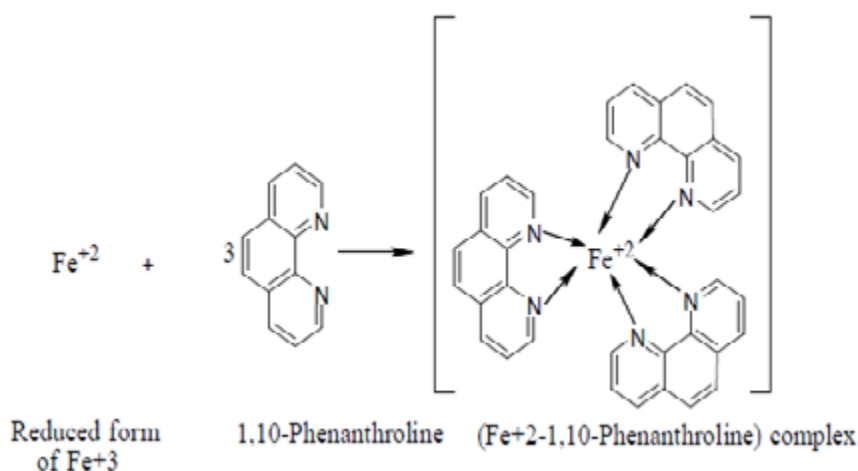
The first step to be taken in the above several proposed methods is the formation of oxidation of PRFX with the oxidant.



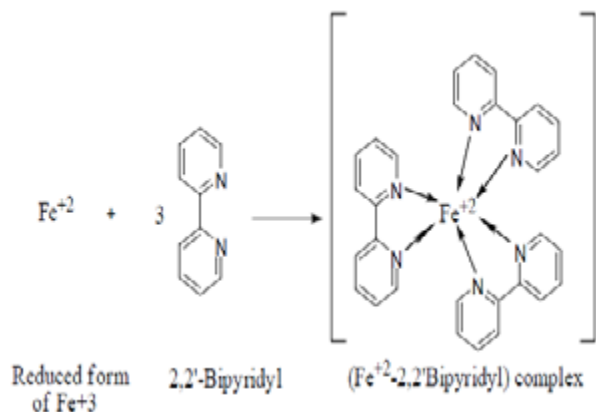
In this method, even though Fe (III) interferes to a little extent in the determination of Fe (II), the reactivity of the interfering entity has to be made insignificant by complexing it with o-phosphoric acid.



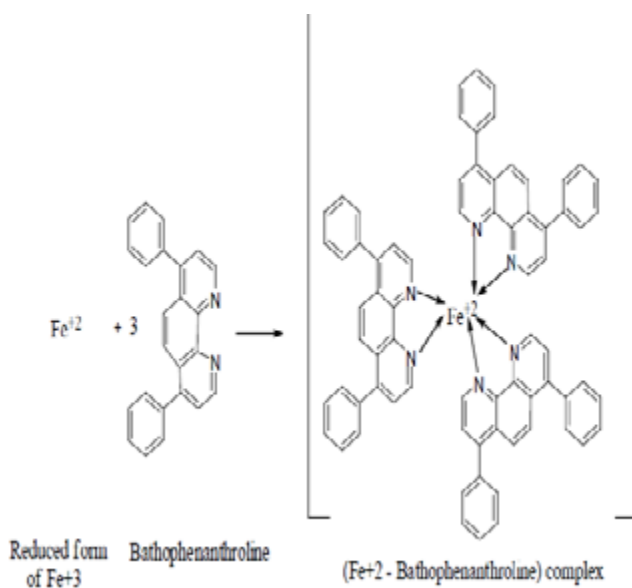
The secondary step connected with the estimation of the decreased form of oxidant with suitable reagents such as 1, 10 - Phenanthroline, 2, 2'- Bipyridyl, Bathophenanthroline is explained in methods M_{5a} , M_{5b} , and M_{5c} . The complex formation of the above explained methods is exhibited in scheme 2 to 4 respectively.



Scheme 2: PRFX reaction with 1, 10- phenanthroline



Scheme 3: PRFX reaction with 2, 2'-bipyridyl



Scheme 4: PRFX reaction with bathophenanthroline

DISCUSSION

In order to ascertain the optimum wavelength of the maximum absorption of the color species formed in each of the five methods specified amounts of PRFX were taken and the colors were developed separately the above said procedures individually. The absorption spectra were scanned on a UV visible spectrophotometer in the wavelength region of 400 - 800 nm against a corresponding reagent blank. The reagent blank absorption spectrum of each method was recorded against appropriate solvent (against 0.1N sodium hydroxide in method M₁, 0.1 N hydrochloric acid in Methods M₂, M₃, M₄; against acetonitrile in methods M_{5a}, M_{5b}, and M_{5c}). The results are graphically represented in Figures 3 to 9. The absorption curves of colored species formed in each method shows characteristic absorption maxima (Table 6). The absorption maxima of M₁, M₂, M₃, M₄, M_{5a}, M_{5b} and M_{5c} were found to be 275, 580, 700, 420, 510, 520 and 600 nm respectively. The beer's law plots of these systems were recorded and graphically exhibited in Figures from 10 to 16. Beer's law limits, molar absorptivity, sandell's sensitivity and optimum

photometric range for PRFX in each developed method with the selected reagents were calculated and presented in Table 6. The % RSD values were found to be less than two, which indicates that all the developed methods were precise. Least square regression analyses was conducted and got slope, intercept and correlation coefficient values which are shown in Table 6. Regarding precision the percent relative standard deviation and percent range of error (confidence limits P = 0.05 and 0.01 levels) were computed and they were explained in Table 6. Recovery studies were conducted by analyzing each pharmaceutical formulation in the first instance for the active ingredients by recovery examination was taken up by the proposed method. Three different quantities of pure drug were added to the previously analyzed and reported formulations and the total quantity of the drug was once more determined by all the proposed methods after bringing the concentration of active ingredient within the purview of Beer's law limits. The results so obtained were mentioned in Table 6. It was also found that the excipients and additives such as microcrystalline cellulose, magnesium stearate, lactose monohydrate, povidone, sodium starch glycolate, and colloidal silicon dioxide existed in the preparation of PRFX tablet formulation did not interfere while determining the PRFX by the proposed methods even if they are existed in huge quantities than normally present. To find out the suitability of the proposed methods for the assay of pharmaceutical formulations (Tablets) containing PRFX were analysed by each proposed method and reference method. The results obtained from each of the proposed and reference methods were compared statistically by the t - and F - tests and were found that these proposed methods do not differ significantly in precision and accuracy from reference method. The results are tabulated in Table 7.

CONCLUSION

There were no visible spectrophotometric methods have been reported so far for the determination of PRFX in bulk as well as pharmaceutical formulations. Therefore, one UV and four visible spectrophotometric methods have been developed depending on the reactivity of varied structural units for instance piperazine ring and tertiary amine and carbonyl group in PRFX. Each method utilizes a specific reagent and the ϵ_{\max} values of each method are found to be different. The order of sensitivity of the said proposed methods are M₁ > M₂ > M_{5c} > M_{5b} > M_{5a} > M₄ > M₃. It is observed that the statistical analysis results of correlation coefficient of each method values are observed to be greater than 0.999 which speaks that the proposed methods have good linearity. The % RSD values are observed below two, which shows that all the proposed methods are precise. The results of analysis of the pharmaceutical formulations assert that the proposed methods are aptly suitable for their estimation without interference of the usual excipients and additives. Therefore these methods can safely be adopted for routine quality control of PRFX in bulk and

pharmaceutical preparations to yield correct and accurate analysis results.

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