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ESTIMATION OF IPRATROPIUM BROMIDE BY EXTRACTION FREE SPECTRO-PHOTOMETRIC METHOD USING SULPHONAPTHALEIN DYE

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Keywords:

Anticholinergic, Ipratropium Bromide, Bromophenol blue, Spectrophotometry, Complex formation, Pharmaceutical analysis

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ABSTRACT: The present paper portrays a simple, rapid, nonextractive spectrophotometric method for the estimation of an anticholinergic drug, Ipratropium Bromide. The method is based on the formation of an instantaneous stable yellow colored ion pair complex of drug with a chloroformic solution of reagent Bromophenol Blue (BPB) which shows absorption maxima at 416 nm. Job's plot of continuous variation affirmed the development of 1:1 complex between drug and reagent. The optical parameters were optimized, and the calibration curve was found to be linear in the range of 10 µgmL^{-1} to 80 µgmL^{-1}. The detection limit was found to be 2.75 µg mL^{-1} with Sandell's sensitivity 0.083 µgcm^{-2}. Stability constant (log K_f) was calculated as 6.6. Molar absorptivity was found to be 4.29 × 10^3 L mol^{-1} cm^{-1} with Gibb's free energy change of - 3.1 × 10^4 kJ mol^{-1}. The method was validated as per ICH guidelines and had many advantages like low detection limits, high sensitivity and not requiring adjustment of critical conditions, thus paving the way for the application of the proposed methods in routine quality control evaluation.

INTRODUCTION: Ipratropium bromide (IPB) is a congener of atropine obtained by treating atropine with isopropyl bromide [1]. It was designed as an orally active, profoundly polar drug which ineffectively infiltrates the blood-brain barrier, thus preventing the central side effects (anticholinergic syndrome). Chemically Ipratropium bromide is 8-Azabicyclo [3,2,1] octane, 3-(3-hydroxy-1-oxo-2-phenylpropoxy)-8-methyl-8-(isopropyl)-, bromide [2]. It antagonizes the action of acetylcholine by blocking muscarinic cholinergic receptors without specificity for subtypes, thus promoting the degradation of cyclic guanosine monophosphate (cGMP) which in turn prevents the increase in intracellular concentration of calcium, restraining bronchoconstriction and mucus secretion.

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The only noteworthy side effect of Ipratropium bromide is dry mouth, at doses higher than the therapeutic dose [3]. It is administered as a unit dose nebulization solution and metered dose inhalers (MDI) for the treatment of obstructive airways disease and allergic rhinitis [4].

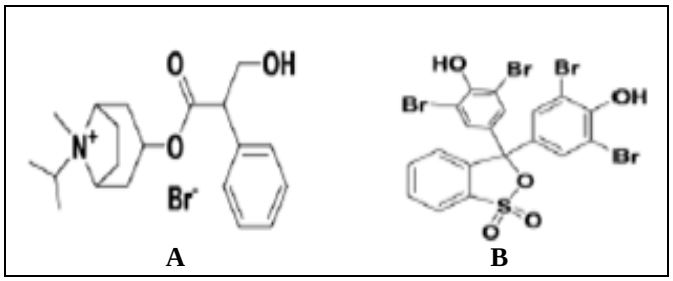

FIG. 1: STRUCTURES OF A. IPRATROPIUM BROMIDE B. BROMOPHENOL BLUE

Bromophenol Blue (BPB) is 3′,3″,5′,5″-tetrabromo phenolsulfonphthalein and is prepared by slowly adding excess bromine to a hot solution of phenolsulfonphthalein in glacial acetic acid and has varied applications like pH indicator, color marker, and dye [5]. Its use as an analytical reagent for estimating drugs has been exploited by a few

researchers [6, 7, 8]. The structure of IPB and BPB is given in **Fig. 1**.

Screening of the writings uncovered that IPB was determined by very few methods. Hassan *et al*., [9] broadened the utilization of derivative spectroscopy for its estimation, but, yet derivative methods do suffer from the disadvantage that it is instrument particular. IPB was determined in aerosol or liquid for nebulization using HPLC [10, 11]. Other HPLC methods quantified IPB and its related substances [12, 13]. A radioreceptor assay method looked into the levels of IPB in biological fluids of human plasma and urine [14]. A non-aqueous titration was likewise detailed. The official methods are reported in BP, EP, and Japan Pharmacopoeia.

European Pharmacopeia (5th Edition) measured IPB potentiometrically by assay of a pure substance with silver nitrate [15] while BP (1998) utilized an HPLC method for the assay of the drug in liquids for nebulization [16]. The other spectrophotometric methods reported were based on the oxidation of IPB with alkaline $KMnO_4$ [17]. Spectrophotometric methods reported depended on complexation of drug with Iodine and Bromocresol green [9]. However, the reported methods, particularly those based on chromatography are complex; requiring expensive experimental setup and skilled personnel, making it inaccessible to many laboratories in developing and underdeveloped nations.

In contrast, visible spectrophotometry is considered as the most convenient analytical technique in most quality control and clinical laboratories. The spectrophotometric methods reported utilized principles of extraction spectrophotometry, requiring a tedious liquid-liquid extraction step, rigid pH control; in this way trading off selectivity and detection limits as cited in **Table 5**.

Hence, there is a need to develop a simple, precise, sensitive, cost-effective extraction free spectro-photometric method of analysis for the estimation of IPB which can be carried out even in small-scale laboratories for routine daily analysis.

EXPERIMENTAL:

Apparatus: Shimadzu 1800 double beam UV-Visible spectrophotometer was used to record the spectra of individual components as well as the ion-pair complex, using a matched pair of Quartz cells of 10 mm path length connected to an IBM computer loaded with the UVPROBE 2.43 application software. A calibrated electronic balance was used for weighing purposes. Glassware's were thoroughly cleaned before the conduct of the study.

Materials: Ipratropium Bromide (CAS No-18559-94-9) was purchased from Vamsi Laboratories, Sholapur, India. Bromophenol Blue, Methanol, Ethanol, Acetone, Dichloromethane and Chloroform were obtained from Rankem Laboratories, Mumbai, India. All chemicals and reagents used were of analytical grade and were used as such without further purification.

Reagent Solution: 0.1% w/v (1mg mL^{-1}) solution of Bromophenol Blue was prepared by accurately weighing and carefully transferring 100 mg of the reagent in 100 ml volumetric flask, and the volume was made up with chloroform.

Standard Drug Solutions: A Stock solution containing (1000 µg mL^{-1}) of IPB was prepared by accurately weighing and carefully transferring 100 mg of the drug into 100 ml volumetric flask. Then 1.0 ml methanol was added, and the resultant mixture was sonicated for about 5 min till the complete dissolution of the drug. The resultant solution was made up to volume with chloroform.

General Procedure for the Spectrophotometric Method: A suitable aliquot of the standard working solutions of IPB was accurately transferred into a series of 10 mL volumetric flasks so that the final concentration was in the range of 10-80.0 µg mL^{-1}. To it, 0.5 mL of 0.1% BPB solution was added. The contents were mixed well, and chloroform was used to complete the volume. The absorbance of the resulting chloroformic solutions was measured against a reagent blank treated similarly at 416 nm.

Procedure for Sample Preparation from Marketed Formulation: Commercial respules (IPRAVENT) each containing (0.5 mg of Ipratropium in 2 ml of solution) was purchased from the local market and the contents of twenty vials were squeezed into a beaker and the contents were evaporated to dryness to remove the aqueous vehicle and the residue was extracted with

chloroform. Aliquots of IPB was placed in 10 mL volumetric flask and 0.5 mL of BPB was added and the volume was made up to 10 mL with chloroform to get a solution containing 10 µg mL^{-1} and 20 µg mL^{-1} of IPB. The obtained solution was passed through a filter paper containing anhydrous sodium sulfate. The resulting solution absorbance was recorded against reagent blank at 416 nm.

Procedure for Stoichiometric Relationship: The stoichiometric relationship between the drug and the reagent was studied by applying the method of Job's plot of continuous variations. Equimolar solutions 1×10^{-3} M each of both IPB and BPB solutions in chloroform was prepared separately. A series of solutions were prepared in which the total volume of IPB and dye was kept at 10 mL. The drug and dye solutions were mixed in various complementary proportions (1:9, 2:8, 3:7, and 10:0, inclusive) and completed as directed under the recommended procedures by measuring the absorbance of the resultant ion-pair complex at 416 nm [18].

Calculation of Conditional Formation Constants (K_f) and Gibbs Free Energy: The conditional formation constants (K_f) of the ion-pair complex for the studied drug were calculated from the continuous variation plot data [19, 20]. The following equation was used when Drug –Reagent Complex is in the ratio 1:1.

$$K_f = \frac{A/Am}{\left(1-\frac{A}{Am}\right)^{n+2} Cm^n} \quad \ldots\ldots (1)$$

Where; A = Max. Absorbance;
Am = Absorbance corresponding to the intersection of the two tangents of the job plot;
C = Molar concentration of drug corresponding to maximum absorbance.

The standard free energy change of complexation (ΔG) was calculated using the formula

$$\Delta G = -2.303\ RT \log K_f \ldots\ldots (2)$$

Where ΔG = Gibbs free energy change of the reaction (kJ mol^{-1});
R = Universal gas constant (8.3145 KJ mol^{-1} K^{-1});
T = Absolute temperature (298 K) and K_f = Conditional formation constant of the drug reagent complex.

Validation: The proposed method was validated according to ICH guidelines [21] for linearity, sensitivity, recovery, and precision.

Linearity: Under the above experimental conditions, the calibration curve was constructed by plotting concentration versus absorbance. Linearity was obtained by the method of least squares to generate the regression equation derived from the calibration graph using the equation (4). The statistical parameter correlation coefficient was also calculated.

$$y = mx + c \ldots\ldots (3)$$

Where y = absorbance; m = slope;
c = intercept;
x = concentration in µg mL^{-1} and the data are summarized in **Table 1**.

Sensitivity: The limit of detection and limit of quantitation was calculated according to equations,

$$LOD = 3\sigma / S \ldots\ldots (4)$$

$$LOQ = 10\sigma / S \ldots\ldots (5)$$

The standard deviation of the response is denoted by σ and S is the slope of the calibration graph. The other sensitivity parameters like molar absorptivity, Sandell's sensitivity are calculated as per guidelines and summarized in **Table 1**.

Precision: The concentration of IPB 20, 40, 60 µg mL^{-1} was used to evaluate the precision of the method by determining the repeatability three times on the same day (intraday) and similarly same concentrations were analyzed three times on three different days (interday). The precision data are shown in **Table 2**.

Accuracy/Recovery Studies: Preanalysed Ipravent Respule solution was spiked with pure IPB at three concentration levels (50,100 and 150%), and the total amount of IPB was found by the proposed methods. Special precautions were taken in sample preparation. The mean % recoveries were calculated, and % RSD was tabulated in **Table 3**.

Specificity: Specificity checks any possible interference by the excipients used. It was checked by the use of a placebo blank of commonly employed respule excipients. An aliquot of the isotonic solution was used, and its solution was

prepared as described under the above procedure and analyzed to check the specificity of the method.

Robustness and Ruggedness: The robustness of the method were evaluated by making small, deliberate changes in the analytical parameters like reaction time (5, 10, 15 min), and the changes of the absorbance of the ion-pair complex were studied. Method ruggedness was demonstrated by a single analyst performing analysis using two different cuvettes and also by having the analysis done with the help of two analysts.

RESULTS AND DISCUSSION:

Background: A thorough study of the literature revealed that IPB was estimated by extraction spectrophotometry, as stated earlier. As far as our knowledge was concerned, there was no method reported for the estimation of IPB using BPB as a reagent to complex with, either by extractive or non extractive technique. The knowledge obtained from similar studies used for quantification of drugs with the help dyes formed the backbone of this research [22, 23, 24].

Optimization of Conditions: The method was developed by performing various trials by optimizing parameters like the choice of solvent, the concentration of reagents, and point of time to measure the formed complex.

Choice of Organic Solvent: Solvents like methanol, ethanol, acetone, dichloromethane, and chloroform were used to check the complex formation and its molar absorptivity. Our trials started initially by studying the effects of methanol on IPB: BPB complex solubility. Non-reproducibility of the method was the major problem when methanol was used as a solvent. The varied concentration of BPB and drug failed to show a significant difference in the absorption spectra. The color of the complex formed exhibited a transition from yellow to blue and then to red with varying levels of BPB. Further trials, performed using other solvents like ethanol, acetone, and dichloromethane also proved futile. Although they developed stable color complexes, absorbance spectra revealed major problems like high absorbance value for the reagent blank; noise, negative absorbance and increased fluctuations in the absorbance values for the drug-dye complex peaks. In a nutshell, the polar solvents were found to be unsuitable, as their blanks with BPB, gave high absorbance values, in addition to the low stability constants for complexes formed. Among the various solvents tried chloroform served as a promising solvent as it exhibited negligible blank absorbance and the drug - reagent complex formed showed high molar absorptivity, exhibiting a highly stable and sensitive complex which absorbed at 416nm.

Absorption Spectra: The absence of conventional UV method of analysis in literature is justified by its low UV absorption. IPB is a drug devoid of structural conjugation [25], hence can be estimated by spectrophotometer only after derivatization. The spectrophotometric method is based on the formation of the colored complex (ion-pair or charge transfer) where opposite electric charged drug and reagent are held together by Coulomb attraction in solution to form a distinct chemical entity. The chloroformic solution of sulphonphthalein dye acts as the chromogenic reagent. Upon interaction of IPB with BPB in chloroform, a stable yellow colored ion-pair complex was instantaneously formed which shows maximum absorbance at 416 nm, when recorded against the reagent blank treated similarly. The reagent, as well as the drug, showed negligible absorbance while the complexes showed maximum absorbance at this wavelength. Hence, it was concluded that quantitative analysis of drug could be carried out at this wavelength. The proposed mechanism of the reaction is depicted in **Fig. 2**.

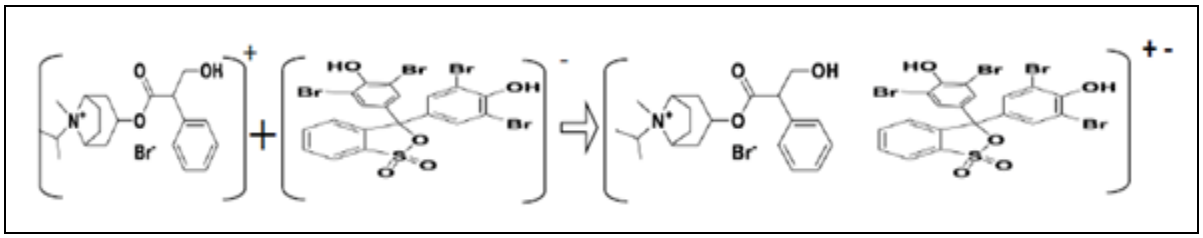

FIG. 2: SCHEMATIC REPRESENTATION OF ION PAIR COMPLEX OF IPRATROPIUM BROMIDE

Effect of Reagent Concentration: The concentration of IPB was fixed and different amounts of reagent (0.1% w/v BPB) were added and resulting absorbance was recorded to study the effect of reagent concentration on the formed complex. The color intensity was found to increase with the addition of BPB up to a particular concentration and then either decrease or steadily remain. The maximum color intensity was obtained with 0.5 mL. Hence 0.5 mL of 0.1% w/v chloroformic solution of BPB was used for the recommended procedure. The effect of reagent concentration is shown in **Fig. 3** and **4**.

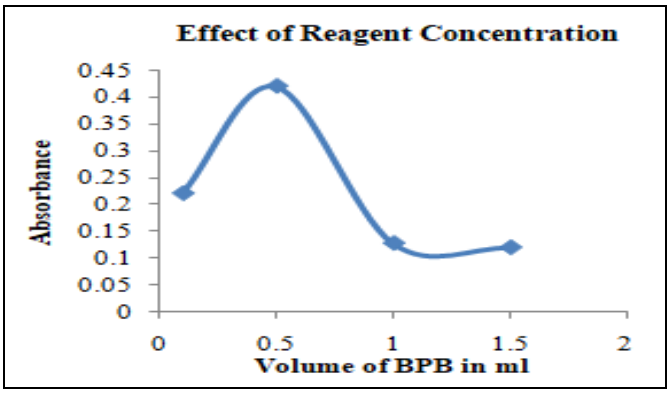

FIG. 3: EFFECT OF VOLUME OF REAGENT – BPB (0.5 ml)

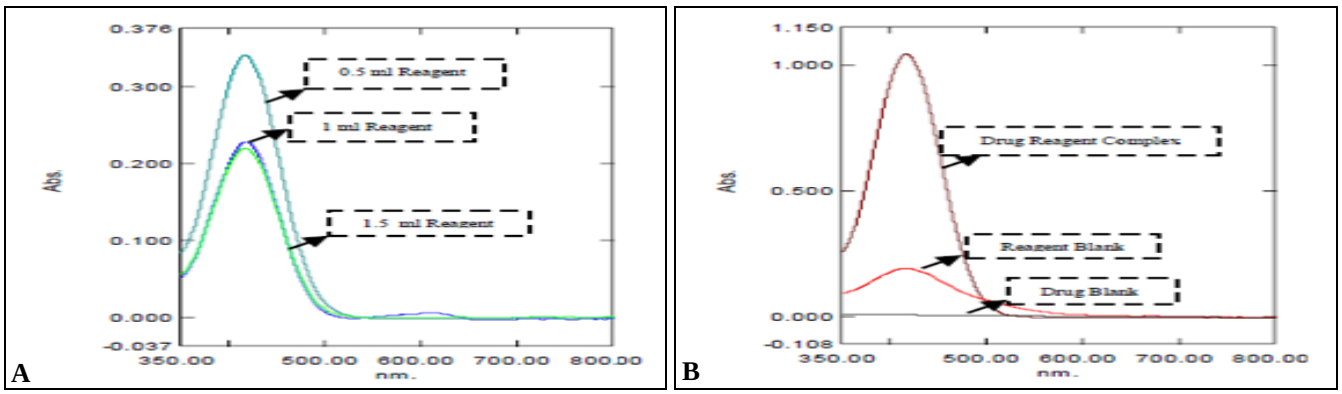

FIG. 4: A. ADDITION OF 0.5 ml REAGENT SHOWED MAXIMUM ABSORBANCE B. REAGENT AND DRUG SHOWED NEGLIGIBLE ABSORBANCE WHILE COMPLEX SHOWED MAXIMUM ABSORBANCE

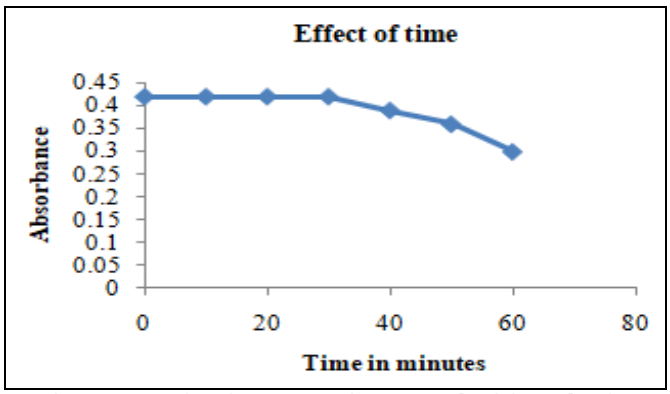

FIG. 5: EFFECT OF TIME ON THE ABSORBANCE OF ION PAIR COMPLEX OF IPRATROPIUM BROMIDE (40 µg mL^{-1})

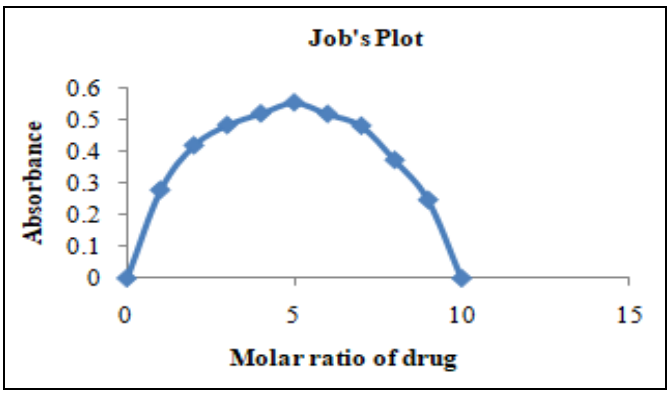

FIG. 6: JOBS PLOT OF CONTINUOUS VARIATION OBTAINED FOR 1 × 10^{-3}. M IPB-BPB ION PAIR COMPLEX

Effect of Time: At different time intervals absorbance of the formed complexes was recorded to understand the optimum reaction time. The variation has been shown in **Fig. 5**. It was found that the complexes were formed instantaneously at room temperature. The absorbance was found to be steady even after 30 min. To remove the time effect, all observations were made after 10 min of complex formation. Precautionary measures were taken to avoid evaporation losses.

Stoichiometric Relationship and Stability Studies: The Job's method of continuous variation was applied to study the stoichiometric relationship and stability constant of the formed complex. The absorbance of each mixture was recorded by mixing equimolar solutions of the drug and the reagent in various proportions. The results indicated that the complex formed was in the ratio 1:1 (D: R), which describes the formation of the early intermediate outer complex. The proposed structure of the drug-reagent complex is shown in **Fig. 2**. The stability constant (log K_f) values were found to be 6.6 showing the high stability of the complex formed. Gibb's free energy was found to be -3.1×10^4. The large negative value of Gibb's free energy is a depiction of the spontaneity of the process (Results recorded in **Table 1**). The continuous variation plot is noted in **Fig. 6**.

Validation:

Linearity: Linearity is the ability of the analytical method to obtain test results that are directly proportional to the concentration of the analyte in the sample. A linear absorbance-concentration correlation was found to be 10-80 µg mL^{-1} with correlation coefficients 0.999. The regression equation was found to be; $y = 0.9934 x + 0.02548$ ……. (7) Where x is a concentration of unknown in µg mL^{-1}. The analytical parameters are recorded in **Table 1**.

Sensitivity: The molar absorptivity and Sandell's sensitivity were found to be 4.293×10^3 L mol^{-1} cm^{-1} and 0.0826 µg cm^{-2}, respectively. Values of molar absorptivities and Sandell's sensitivities reflect the high sensitivity of the method. The LOD and LOQ values are summarized in **Table 1**.

TABLE 1: REGRESSION AND ANALYTICAL PARAMETERS

S. no.	Analytical Parameters	IPB-BPB complex
1	λmax (nm)	416 nm
2	Molar ratio (D:R)	1:1
3	Beer's Law Limits (µg mL^{-1})[b]	10-80
4	%RSD (at 10 µg mL^{-1})	1.65
5	LOD (µg mL^{-1})	2.74
6	LOQ (µg mL^{-1})	8.32
7	Molar absorptivity (L mol^{-1} cm^{-1})	4.293×10^3
8	Sandell's sensitivity [a] (µg cm^{-2})	0.083
9	Stability constant (log K_f)	6.6
10	ΔG (kJ mol^{-1})	-3.1×10^4
	Regression equation Y [c]	
1	Intercept (a)	0.0285
2	Slope (b)	0.0099
3	Correlation coefficient (r)	0.999
4	The standard deviation of intercept (Sa)	±0.0067
5	The standard deviation of the slope (Sb)	±0.0002

[a] Limit of determination as to the weight in lg per ml of solution, which corresponds to an absorbance of A=0.001 measured in a cuvette of cross-sectional area 1 cm^2 and l = 1 cm. [b] average of three determinations. [C] Y=a + bX, where Y is the absorbance, a is the intercept, b is the slope and X is the concentration in l µg ml^{-1}

Precision: The precision of the method was calculated in terms of both intraday and interday. RSD (%) were found to be small below 2%, indicating good repeatability and reliability of the proposed method. **Table 2** depicts the results obtained from Intra-day and Inter-day studies.

Recovery Studies: The recovery percentage values ranged from 99.54 to 103.26% with a standard deviation of ±1.967, indicating that the recovery was good and that the co-formulated substance did not interfere in the determination. The results of recovery studies are shown in **Table 3.**

TABLE 2: EVALUATION OF INTRA-DAY PRECISION AND INTER-DAY PRECISION

Drug taken µg mL^{-1}	Intra-day (n=3)		Inter-day (n=3)	
	Amount found µg mL^{-1}[a]	% RSD[b]	Amount found µg mL^{-1}[a]	% RSD
20	19.48 ± 0.36	1.62	19.3 ± 0.41	1.89
40	41.15 ± 0.57	1.30	41.35 ± 0.84	1.91
60	60.24 ± 1.22	1.93	60.14 ± 1.25	1.99

[a] Average of three determinations, [b] relative standard deviation

TABLE 3: RECOVERY STUDY OF IPRATROPIUM BROMIDE

% Recovery levels	Preanalysed sample taken	Pure Drug added µg mL^{-1}	Total amt present	Amount found µg mL^{-1a}	% Recovery
50	10	5	15	15.38	102.53 ± 2.54
100	10	10	20	19.91	99.55 ± 2.53
150	10	15	25	25.82	103.26 ± 3.14

[a] Average of three determinations

Robustness and Ruggedness: The deliberate changes had a negligible influence on the results as expressed as % RSD (<2). Intermediate precision values in both instances were in the range 0.93 to 1.81, indicating acceptable ruggedness. These results are presented in **Table 4**.

TABLE 4: METHOD ROBUSTNESS AND RUGGEDNESS

Method robustness		Method ruggedness			
Parameters altered Reaction time		Inter-analysts		Inter-cuvettes	
		Analyst 1	Analyst 2	Cuvette 1	Cuvette 2
Conc. µg mL^{-1}	RSD (%)	RSD (%)	RSD (%)	RSD (%)	RSD (%)
20	1.03	1.782993	1.58	1.81	0.93
40	0.823	1.21087	1.61	1.08	1.17
60	0.467	1.560461	1.37	1.04	1.06

[a] The reaction time was 5, 10 and 15 min

Application to Pharmaceutical Formulations: The proposed methods have been successfully applied for the determination of IPB in their pharmaceutical formulation such as respules. Quantitative analysis was performed using the data obtained from the calibration curve. Results obtained recorded in **Table 3** reveals satisfactory assay results which were in a good agreement with the label claims. A comparison of the developed method with reported methods has also been presented in **Table 5**.

TABLE 5: COMPARISON OF PRESENT WORK WITH REPORTED METHODS

Analytical Parameters	Proposed Method	Iodine [9]	Alkaline $KMno_4$ [17]	Bromocresol green [9]	Derivative Spectroscopy [9]
Methodology	Extraction free ion pair complex formed using BPB	Charge transfer complex with Iodine	Oxidation of IPB with alkaline $KMnO_4$	Ion - association complex between the drug and an acidic dye, Bromocresol Green (BCG),	Principles of derivative spectroscopy
λmax (nm)	416 nm	278 nm	608 nm	418 nm	D_2 at 232 nm
Linear range (µg mL^{-1})	10-80	1-10	4.6×10^{-6} – 2.3×10^{-5} M	2-16	5-30
Slope	0.0099	0.0924	-35630.43	0.0490	0.2062
Intercept	0.0285	-2.34 × 10-3	-0.0107	-0.0154	0.1905
Recovery	99.54 to 103.26%	99.67 ± 0.79	100.0191.16	99.26 ± 1.06	100.21 ± 0.85
Correlation coefficient	0.999	0.9999	0.9995	0.9998	0.9999
%RSD	1.65	0.85	NR	1.15	1.15
Molar absorptivity (L mol^{-1} cm^{-1})	4.293×10^3	3.9×10^4	NR	2.1×10^4	NR
LOD (µg mL^{-1})	2.74	0.17	0.35	0.25	0.22
Remarks	Extraction Free, Simple, rapid, sensitive, no heating step and use of single reagent		The method is complex, involving preparation of other reagents	The tedious method involving extraction which is pH dependent	Derivative methods are instrument specific

NR: Not reported

CONCLUSION: The proposed method was based on the formation of drug-reagent complex and had many advantages like low detection limits, high sensitivity, and stability with a wider dynamic range. An important feature of the proposed method is that it is extraction-free requiring only dye and the solvent used was comparatively cheaper and readily available.

The present method does not involve adjustment of critical conditions like temperature, pH, or tedious sample preparation since complex formation is

instantaneous at room temperature. These advantages pave the pathway for the application of the proposed methods in routine quality control evaluation.

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CONFLICT OF INTEREST: There is no conflict of interest.

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