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Research Article

ENZYMATIC ASSAY OF SALBUTAMOL IN BULK AND TABLET DOSAGE FORMS

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ABSTRACT

Enzymatic methods for determining compounds are a key technology in quantification of various analytes of chemical and biochemical interests have found wide range of applications in clinical diagnosis, medical treatment, biochemical research and industrial purposes because of their high specificity and rapidity. The present work is an attempt to develop three novel spectrophotometric methods for the determination of salbutamol in bulk and in its pharmaceutical dosage forms. The proposed methods are based on the oxidative coupling of salbutamol with 3-Methylbenzothiazoline-2-one hydrazone (method M1), Aniline (method M2) and 4 – Aminoantipyrine (method M3) in the presence of hydrogen peroxide and horseradish peroxidase to produce a colored complex having absorption maxima at 450 nm, 480 nm and 490 nm, respectively. The reaction conditions were optimized to obtain maximum color intensity. The absorbance was found to increase linearly with increasing the concentration of salbutamol; the systems obeyed the Beer's law in the range 2–12 µg/ml for methods M1 & M2 and 5–30 µg/ml for method M3. The correlation coefficient values were found to be 0.9985 (M1) and 0.9987 (M2 & M3). Sandell's sensitivity is calculated as 0.02247, 0.00943 and 0.04950 µg/cm^2/0.001 abs. unit for methods M1, M2 and M3, respectively. Results of analysis of these methods were validated statistically and by recovery studies. The method is applied to the marketed tablet formulation. The percentage relative standard deviations are 0.568-0.884, 0.493-0.713 and 0.503-0.692 for methods M1, M2 and M3, respectively. The accuracy was examined by performing recovery studies and was found to be 97.25-103.0 (M1), 99.0-101.25 (M2) and 97.50-102.25 (M3). No interference was observed from common excipients present in pharmaceutical formulations. The developed methods are simple, sensitive and reproducible and can be used for routine analysis of salbutamol in bulk and tablet dosage form.

KEY WORDS: Horseradish peroxidase, salbutamol, coupling agents, spectrophotometric method, validation.

INTRODUCTION

Salbutamol (SBL), chemically known as 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl) phenol (Figure. 1), is a short-acting β_2-adrenergic receptor agonist. It is used for the relief of bronchospasm in conditions such as asthma[1,2] and chronic obstructive pulmonary disease[3,4]. In low doses it acts relatively selectively as β_2-adrenergic receptor to cause bronchodilation and vasodilation; at higher doses, β2 selectivity is lost, and the drug acts at β-2 receptors to cause typical sympathomimetic cardiac effects. The smooth muscles are relaxed by the SBL by the increase in the intracellular cyclic adenosine monophosphate[5,6,7]. Because of this there is relaxation in bronchial and uterine muscles; also there is increase in heart rate and dilation of the peripheral vessels. ATPase[8] channels are opened by the activation of the β-2 adreno-receptors which inturn drives potassium from the extra cellular to the intracellular space. This results in a decrease in extracellular Hyperkalaemia and

increase in intracellular potassium, which decreases the chance of arrhythmias. SBL also has certain anti-inflammatory[9] properties whose clinical significance is not determined.

The therapeutic importance of SBL has prompted several researchers to develop methods for its determination in bulk, pharmaceutical dosage forms and biological fluids. The drug is official in European Pharmacopoeia[10], British Pharmacopoeia[11] and Indian Pharmacopoeia[12]. British pharmacopoeia describes a potentiometric titration in non aqueous medium for the determination of drug. The methods used for SBL assay include UV spectrophotometric[13-15], HPLC with UV detection[16-20], HPLC with fluorescence detection[21], isotachophoresis[18], capillary zone electrophoresis[18], immunoaffinity chromatography[22], thin layer chromatography[23], LC-MS/MS[24,25], supercritical fluid chromatography[26], spectrofluorimetric[27], conductometric[28], titrimetric[29-31] and spectrophotometric methods[29-37]. Most of these methods (except titrimetric and spectrophotometric) require expensive or sophisticated instruments or involve procedures with rigorous control of the experimental conditions and are not simple for routine analysis. However, spectrophotometric methods are particularly attractive because of ease in accessibility and their quick applicability to routine analysis. Many researchers introduced various reagents for spectrophotometric determination of SBL in bulk and pharmaceutical dosage forms such as Folin ciocalteau reagent[32,33], Cerium(IV)/MBTH[34], $NaNO_2$/phenylhydrazine sulphonic acid[35], HNO_3/H_2SO_4[36], 2,6-dichloroquinonechlorimide[37], 7,7,8,8-tetracyanoquinodimethane[37], *N*-bromosuccinimide/rhodamine-B[30] , *N*-bromosuccinimide/methylene blue[30], bromate-bromide mixture/rhodamine B[29] and bromate-bromide mixture/methylene blue[29]. Most of the procedures suffer from one or more disadvantages like extraction, less sensitivity, heating, boiling for about 20-30 minutes, strong acid medium, low range of determination, use of organic solvents, indirect determination and critical working conditions. Hence they are not employed for routine analysis.

Conductometric titration method was developed by Issa et al[28] for the determination of SBL by using phosphotungstic acid and phosphomolybdic acid as acid titrants. Basavaiah etal reported two indirect titration methods, based on the determination of surplus *N*-bromosuccinimide[30] and bromine[29] iodometrically after allowing the reaction between SBL and *N*-bromosuccinimide & bromine. Titrimetric method reported by N. Geetha et al[31] employs *N*-bromosuccinimide as the oxidimetric titrant in the presence of potassium bromide and using methyl red as indicator. Even these reported titrimetric methods are time consuming, less sensitive, indirect and applicable over a macro scale.

Peroxidases are widely distributed enzymes that have a considerable scope for undertaking potentially useful transformations in organic reactions. As peroxidase catalyzes the oxidation of a variety of electron donors and these oxidized donors can be condensed with a number of coupling reagents, which produced more complex colored species. The concentration of resulting colored complex indicates that the amount of substrate can be measured spectrophotometrically. The oxidative coupling reactions catalyzed by HRP have many analytical applications in chemistry and biochemistry[38-48]

The literature on enzymatic assay of SBL in bulk and tablet dosage forms is very poor. The present paper, for the first time, describes three novel, sensitive, simple, accurate and precise enzymatic methods for the determination of SBL in bulk and pharmaceutical dosage forms. The proposed methods are based on the oxidative coupling reaction between SBL and coupling agents, 3-Methylbenzothiazoline-2-one hydrazone (method M1), Aniline (method M2) and 4-Aminoantipyrine (method M3) in the presence of horse radish peroxidase (HRP) and hydrogen peroxide. The results of the analysis were validated by statistical analysis and recovery studies according to International Conference on Harmonization (ICH)[49] guidelines for validation of analytical procedures. Common additives used as excipients in the pharmaceutical dosage forms do not interfere in the determination of the SBL.

MATERIALS AND METHODS

Apparatus

1. An ELICO Model SL-159 double beam, UV-VIS spectrophotometer with 1.0 cm matched quartz cells was used for all absorbance measurements.
2. Hydrogen ion concentration (pH) of the solutions was determined by Systronics digital pH meter.

3. Extraction of peroxidase from horseradish was done by using Remi desktop centrifuge with 24,000 rpm.
4. Homogenizer with a high speed blender 3-4 x 15 sec. was used for homogenization of horseradish root.

Preparation of Reagents

All chemicals were of analytical reagent grade. Double distilled water was used to prepare all solutions

For Assay of Salbutamol

1. 0.2% (w/v) 3-Methylbenzothiazoline-2-one hydrazone (MBTH): Prepared by dissolving 200 mg of 3-Methylbenzothiazoline-2-one hydrazone (Himedia lab pvt Ltd, Mumbai, India) in 100 ml of distilled water.
2. 1% (v/v) Aniline (ALN): Dilute 1 ml of aniline (Merck, Mumbai, India) to 100 ml with distilled water
3. 2% (w/v) 4-Aminoantipyrine (4-AP): Prepared by dissolving 2 gm of 4-amino antipyrine (s.d. Fine-Chem Ltd, Mumbai, India) in 100 ml of distilled water.
4. 0.01M Hydrogen peroxide (Merck, Germany) was prepared by dissolving 0.1 ml of 30% hydrogen peroxide in 200 ml of distilled water just prior to experiments.
5. 0.1 N HCl: 0.365 ml of HCl (Merck, Mumbai, India) was added to 100 ml of distilled water to get 0.1 N HCl.
6. 0.1M Phosphate buffer (pH-7.0) was prepared as follows.
   Stock Solutions for buffer:
   a. 0.5 M Potassium dihydrogen phosphate (s.d. Fine-Chem Ltd, Mumbai, India):
   Dissolve 68.04 g of KH_2PO_4 in 1 liter of reagent grade distilled water.
   b. 0.5 M Disodium hydrogen phosphate (s.d. Fine-Chem Ltd, Mumbai, India):
   Dissolve 71g of Na_2HPO_4 in 1 liter of reagent grade distilled water.
   39 ml of 0.5 M KH_2PO_4 and 53.6 ml of 0.5 M NaH_2PO_4 were diluted to 1000 ml at 25^0C.

For Assay of Horse radish peroxidase

1. 0.018 M Guaiacol: Aqueous solution of Guaiacol (Merck, Germany) was prepared by dissolving 223 mg of guaiacol in 100 ml of distilled water. Store on ice and prepare fresh daily.
2. Substrate solution: Dilute 0.1 ml 30% hydrogen peroxide (Merck, Germany) with distilled water to 120 ml and adjust absorbance at 240 nm in 1 cm light path to 0.4 to 0.41 versus distilled H_2O. Store the solution on ice and prepare fresh daily.
3. Potassium Phosphate Buffer (pH 7.0): Dissolve 0.53 gm KH_2PO_4 (s.d. Fine-Chem Ltd, Mumbai, India) and 1.06 gm K_2HPO_4 (s.d. Fine-Chem Ltd, Mumbai, India) in distilled H_2O, check pH to 7.0 and dilute to 100 ml. Store buffer on ice and equilibrate buffer at 25°C.

Preparation of standard drug solution

Salbutamol pure drug was obtained as a gift sample from local pharmacy industry. Pharmaceutical dosage forms Asmanil (Inga, Jaipur), Asthanil (Cipla LTD, Mumbai) and Brethmol (standard, Ahmedabad) containing 2 mg of salbutamol were purchased from local pharmacy market and were employed in the present investigation.

A stock standard solution of SBL (1 mg/ml) was prepared by dissolving pure SBL in double distilled water. Working standard solutions were prepared by suitable dilution of the stock standard solution with double distilled water to get a concentration of 100 µg/ml for the methods M1, M1 and M3.

A stock standard solution of SBL (1 mg/ml) was prepared by dissolving pure SBL in 0.1 N HCl. Working standard solutions were prepared by suitable dilution of the stock standard solution with the same solvent to get a concentration of 500 µg/ml for the reference method.

Extraction and assay of the enzyme (Horseradish Peroxidase)

A turnip (Horseradish root) weighing 40 g was peeled, washed, and cut into 1" cubes. The sliced pieces were homogenized in 200 ml of buffer in a blender at high speed for 15 min. The extract is clarified by centrifugation (10-15,000 rpm/10 min.) and filtered through Whatman No. 1 filter paper. The extract for stability was stored in toluene for at least a week at 4°C. The extract was suitably diluted with

potassium phosphate buffer for further enzyme assay and with 0.1 M Phosphate buffer for experimental analysis.

The assay of extract is based on that of Bergmeyer[50] in which the rate of decomposition of hydrogen peroxide by peroxidase is determined by measuring the rate of color development spectrophotometrically at 436 nm and at 25°C in the presence of guaiacol as hydrogen donor. The enzyme activity is expressed in terms of Guaiacol units (1 Guaiacol unit = Amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C).

General assay procedure

After systematic and detailed study of the various parameters involved as described under results and discussions the following procedures were recommended for the determination of SBL in bulk and tablet dosage forms.

Into a series of 10 ml calibrated flasks, 3 ml of buffer (pH 7.0) solution, 1.5 ml hydrogen donor [0.2 % MBTH (M1), 1 % ALN (M2), 2% 4-AP (M3)], 1 ml of H_2O_2 (0.01 M) and 1 ml enzyme extract (1.6 Guaiacol units) and aliquots of working SBL [0.2-1.2 ml (M1 & M2) and 0.5-3.0 ml (M3)] solution were added and brought up to the volume with buffer. The tubes were incubated at room temperature for 25 min (M1) or 20 min (M2 & M3). The absorbance of the colored chromogen was measured at 450 nm, 480 nm and 490 nm against reagent blank, prepared similarly omitting the drug, for methods M1, M2 and M3 respectively. The amount of the drug in the sample solution was computed from the corresponding calibration graph.

Reference method

Absorption maxima of SBL (20 µg/ml) in 0.1N HCl was determined by scanning the drug solution from 200-400 nm and was found to be at 275 nm. Different aliquots (0.2 – 2.0 ml) of working standard solutions containing 10-100 µg/ml of SBL prepared were transferred into a series of serially numbered 10 ml volumetric flasks. The flasks were diluted to 10 ml with 0.1N HCl. The absorbance of the solution was measured at 275 nm using 0.1N HCl as blank. The amount of SBL present in the sample was computed from the corresponding calibration curve.

Assay procedure for pharmaceutical dosage forms

For the analysis of SBL in pharmaceutical dosage forms, three brands of commercially available tablets were weighed and ground into a fine powder. An accurately weighed portion of the powder equivalent to 100 mg of drug was transferred into a 100 ml beaker containing small volume of water and the solution was shaken thoroughly for 10-15 minutes and filtered through a Whatman filter paper no.1 to remove the insoluble matter. The filter paper was washed with water and the washings were added to the filtrate, the final volume (100 ml) was made with water. This solution was further diluted according to the need and then analyzed following the proposed methods (M1, M2 and M3). The nominal content of drug in the tablets was calculated either from a previously plotted calibration graph or using the regression equation.

RESULTS AND DISCUSSION

Nature of the colored species

The proposed methods are based on the oxidative coupling reaction of the drug, SBL, with coupling agents MBTH (M1), ALN (M2) and 4-AP (M3), in the presence of hydrogen peroxide and HRP to give a colored product. Actually, this is an enzyme catalyzed oxidative coupling reaction of coupling reagent with the drug. Under the reaction conditions, on oxidation by the enzyme in the presence of hydrogen peroxide, coupling agent loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling species. This intermediate undergoes electrophilic substitution with SBL to form the colored product. The absorption spectra of the colored products were taken against reagent blank in the range 380-760 nm. The maximum absorption wavelength was found to be 450 nm, 480 nm and 490 nm for methods M1, M2 and M3 respectively (Figure 2). Under the experimental conditions each colorless reagent blank showed a negligible absorbance at the corresponding λ_{max}. The probable reaction mechanism is given in Schemes 1, 2 and 3.

Optimum conditions fixation in procedures

The optimum conditions for the color development of methods (M1, M2 and M3) were established by varying the parameters one at a time, keeping the others fixed and observing the effect produced on the absorbance of the colored species. The following experiments were conducted for this purpose and the conditions so obtained were incorporated in recommended procedures. The optimized conditions are maintained throughout the experiments to determine the quantity of SBL in bulk and drug formulations.

Effect of pH

All enzymes display a characteristic range of pH at which they are most active. This "pH optimum" may be due to several factors involving the structure and ionic state of the enzyme, substrate, or cofactors. The influence of pH on the development of color was studied using 0.1 M phosphate buffer. Different phosphate buffers with pH range of 3.0 – 9.0 were tried. Variations of the pH less than 6 and greater than 8 resulted in low absorbance values (Table 1). So pH 7 was selected as pH of choice for all the proposed methods.

Effect of temperature

The rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. The reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most enzymes rapidly become denatured at high temperatures. The influence of temperature on the colored product was studied at different temperatures (10-80^{0}C). It was observed that the obtained colored products in the proposed methods (M1, M2 and M3) were stable up to 25-30^{0}C. However, no considerable improvements were occurred above 30^{0}C therefore 25-30^{0}C was selected as optimum temperature for all the proposed methods (Table 1). All experiments and absorbance measurements were carried out at laboratory temperature (28^0$\pm$3^{0}C). At low temperatures ($\leq$20^{0}C) the time required for attaining maximum color is more. At high temperatures (>35^{o}C) the stability of the colored species is less. So laboratory temperature is preferred for all the proposed methods.

Effect of incubation time

Keeping the other conditions constant, the effect of incubation time for the development of maximum color was studied. The contents of the reaction mixture in all the proposed methods (M1, M2 and M3) were kept at laboratory temperature for up to 45 minutes. Absorbance is determined at various standing time intervals when the reaction temperature is 28^0 ± 3^{0}C. It was found that absorbance is maximal when the standing time is 25 min (M1) and 20 min (M2 & M3) (Table 1). Therefore, the above said incubation times are selected for further experiments.

Effect of Enzyme Concentration

When developing an enzymatic assay, one needs to adjust the enzyme concentration so the reaction rate will be easily and accurately detected. In addition, you must determine if the enzyme extract contains any unknown activators (such as organic or inorganic cofactors) or inhibitors, because these must be controlled or removed if further experiments are to be valid. Assaying various enzyme concentrations under standardized conditions and saturating levels of substrate can meet both of these requirements. The assay of horse radish extract for peroxidase activity was carried by using guaiacol as hydrogen donor and the results are summarized in Table 1.

Effect of concentration of Hydrogen peroxide

The influence of the volume of 0.01 M hydrogen peroxide on the rate of reaction was investigated in the range of 0.5-4.0 ml. The rate of reaction was increased with increasing volume of hydrogen peroxide and became constant at 1.0 ml (for methods M1, M2 and M3). Beyond this volume, the initial rate remained constant. Therefore, the same volume of 0.01 M hydrogen peroxide was recommended for all the determination procedures (Table 1).

Effect of concentration of coupling reagents

Effect of MBTH

The effect of volume of 0.2% MBTH on the color development in the method M1 was investigated over the range 0.5-4.0 ml. The results showed that 1.5 ml of MBTH was sufficient to give maximum intensity of the color.

Effect of ALN

The effect of volume of 1% ALN on color development of the product in method M2 was investigated with varying volumes (0.5-4.0 ml) of 1% ALN. The highest absorbance was obtained with 1.5 ml of 1% ALN. Above this volume, there is no change in absorbance.

Effect of 4-AP

The effect of volume of 2% 4-AP on color development of the product in method M3 was investigated with varying volumes (0.5-4.0 ml) of 2% 4-AP. The highest absorbance was obtained with 1.5 ml of 2% 4-AP. Above this volume, there is no change in absorbance.

The results regarding the effect of concentration of coupling reagents on the development of color in the proposed methods are complied in Table 1.

Stability of colored complex

The stability of the colored complexes in the all the proposed methods was monitored by keeping the solutions at room temperature (28±3^{o}C) for several hours and then recording the absorbance of the complex at their respective λmax. The results are presented in Table 1.

Order of addition of reactants

The author has carried out series of experiments to test whether the variation in the order of addition of reactants effect the absorbance of colored products. The suitable order or addition of reactants in the determination of SBL with coupling reagents and HRP/H_2O_2 for attaining maximum color and stability are represented in Table 2.

Method validation

Validation was carried out by assessing the parameters like linearity range, precision, accuracy, detection and quantification limits according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures.

Linearity

A linear correlation was found between absorbance at λ_{max} and concentration of SBL for all the three methods (M1, M2 and M3). The graphs showed negligible intercept and are described by the equation:

$$\mathbf{Y = a + bX}$$

Where Y = absorbance of 1-cm layer of solution

a = intercept

b = slope

X = concentration of drug in μg/ml

The linearity was evaluated by linear regression analysis of the Beer's law data by least-square regression method, which was used to calculate the correlation coefficient, intercept and slope of the regression line and the values are presented in Table 3. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell's sensitivity values of the proposed methods were calculated and are summarized in Table 3.

Accuracy and Precision

In order to determine the intraday accuracy and precision of the proposed methods (M1, M2 and M3), solution containing fixed concentration (within the working limits) of the drug was prepared and analyzed in six replicates by the proposed methods under the optimized experimental conditions. The standard analytical errors, standard deviation, relative standard deviations and recoveries obtained in the intra day analyses for methods M1, M2 and M3 were calculated and are summarized in Table 3. The relative standard deviation indicates the high intra-day precision of the methods. Regarding the accuracy evaluation, good recoveries were obtained. The percent recovery indicated good accuracy and an agreement between the theoretical value and the real value of concentration. Thus the proposed methods are effective for the determination of SBL.

LOD and LOQ

Sensitivity of the proposed methods was evaluated by calculating Limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest detectable concentration of the analyte by the method while LOQ is the minimum quantifiable concentration. LOD and LOQ were calculated by equations: LOD = δ3.3/s and LOQ = δ10/s, respectively, where δ is the standard deviation of blank and s is slope of calibration. The results (Table 3) indicating proposed methods are highly sensitive.

Recovery studies

The reliability and accuracy of the proposed methods were further confirmed by performing recovery studies by standard addition method. To a fixed and known quantity of the pre-analyzed tablet, pure drug (2 mg) was added and the total was found by the proposed methods (M1, M2 and M3). The results of recovery study are complied in Table 4. The percent recoveries of the pure drug added was quantitative and additionally reveal the fair selectivity of the method.

Application of the proposed methods for the analysis of tablets

The proposed methods (M1, M2 and M3) were successfully applied to the determination of SBL in three different brands of tablet dosage forms. The results are summarized in Table 5. The results obtained were statistically compared with the reference method by applying the Student's t-test and F-test for accuracy, precision respectively. The calculated t-value and F-value at 95% confidence level did not exceed the tabulated values of 2.306 and 6.39, respectively, for eight degrees of freedom. The tests indicate that there is no difference between the proposed methods and the reference method with respect to accuracy and precision.

CONCLUSIONS

Three simple, accurate, precise and sensitive enzymatic methods were developed and validated for the analysis of SBL. The statistical parameters and recovery study data clearly indicate the reproducibility and accuracy of the method. The proposed methods can be used for the routine quality control of SBL in bulk and in its pharmaceutical dosage forms.

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Table 1: Optimization of conditions for the proposed methods

Parameter	Investigation conditions	Conditions in procedure		
		M1	M2	M3
λ_{max} (nm)	380-760	450	480	490
Effect of pH	3-9	7	7	7
Effect of temperature (^{0}C)	10-80	28 ± 3	28 ± 3	28 ± 3
Incubation time (minutes)	0-45	25	20	20
Enzyme concentration (guaiacol units)		1.6	1.6	1.6
Effect of volume of 0.01 M H_2O_2 (ml)	0.5-4.0	1.0	1.0	1.0
Effect of concentration of coupling agent:				
0.2 % MBTH (ml)	0.5-4.0	1.5	---	---
1 % ALN (ml)	0.5-4.0	---	1.5	---
2% 4 -AP (ml)	0.5-4.0	---	---	1.5
Stability of the colored species (hours)		1.5	2.0	1.5

Table 2: Sequence of addition of reactants favoring production of maximum absorbance and stability

Coupling reagent	Drug	Order of addition of reactants	Variance from maximum absorbance
MBTH	SBL	MBTH + H_2O_2 + HRP + SBL H_2O_2 + HRP + SBL + MBTH SBL + H_2O_2 + HRP + MBTH	No variance 18% Decrease 13 % Decrease
ALN	SBL	ALN + H_2O_2 + HRP + SBL H_2O_2 + HRP + SBL + ALN SBL + H_2O_2 + HRP + ALN	No variance 12% Decrease 10% Decrease
4-AP	SBL	4-AP + H_2O_2 + HRP + SBL H_2O_2 + HRP + SBL + 4-AP SBL + H_2O_2 + HRP + 4-AP	No variance 10% Decrease 19% Decrease

Table 3: Optical and Regression characteristics, Precision and Accuracy of the proposed methods

Parameters	Method M1	Method M2	Method M3
λ_{max} (nm)	450	480	490
Beer's law limit (µg/ml)	2 - 12	2 - 12	5 - 30
Sandell's Sensitivity (µg/cm^2/0.001 abs. unit)	0.02247	0.00943	0.04950
Molar absorptivity (Litre.mole^{-1}.cm^{-1})	1.056 x 10^4	2.536 x 10^4	1.483 x 10^4
Optimum photometric range (µg/ml)	2.86 – 10.75	2.95 – 11.35	7.10 – 7.68
Regression equation (Y) [$$]:			
Intercept (c)	0.0014	-0.0037	0.0004
Slope(m)	0.0428	0.1011	0.0198
Correlation coefficient (r)	0.9985	0.9987	0.9987
Limit of Detection (LOD) (µg/ml)	0.188	0.055	0.200
Limit of Quantification (LOQ) (µg/ml)	0.570	0.168	0.606
Standard deviation (SD)[$]	0.0024	0.0017	0.0012
% Relative standard deviation (RSD)	1.223	0.297	1.200
% Range of errors (95% confidence limits):			
0.05 significance level	1.019	0.248	1.003
0.01 significance level	1.509	0.367	1.484
% Error in bulk samples	0.568	0.462	0.753

[$$]Y = mx + c, where Y is the absorbance and x is the concentration of drug in µg/ml. [$]Average of six determinations

Table 4: Recovery of salbutamol by standard addition method

Method	Formulation	Labelled claim (mg)	Pure drug added (mg)	Found ± S.D (n=5)	% Recovery
M1	Asmanil	2	2	3.89 ± 0.346	97.25
	Asthalin	2	2	4.05 ± 0.368	101.25
	Brethmol	2	2	4.12 ± 0.462	103.0
M2	Asmanil	2	2	3.96 ± 0.223	99.0
	Asthalin	2	2	4.01 ± 0.462	100.25
	Brethmol	2	2	4.05 ± 0.589	101.25
M3	Asmanil	2	2	3.90 ± 0.761	97.50
	Asthalin	2	2	3.95 ± 0.462	98.75
	Brethmol	2	2	4.09 ± 0.308	102.25

Table 5: Assay of salbutamol by the proposed methods and reference method

Formulation	Labeled amount (mg)	% Found ± S.D[$]			
		Reference method	Method M1	Method M2	Method M3
Asmanil	2	101.5 ± 0.734	99.00 ± 0.876 % RSD =0.884 t = 0.55 F = 1.17	99.00 ± 0.706 %RSD = 0.713 t = 1.49 F = 2.57	99.50 ± 0.689 %RSD = 0.692 t = 0.56 F = 1.28
Asthalin	2	97.50 ± 0.564	101.50 ± 0.672 %RSD =0.662 t = 0.47 F = 1.03	98.50 ± 0.486 %RSD =0.493 t = 2.19 F = 3.18	102.50 ± 0.614 %RSD =0.599 t = 0.48 F = 1.97
Brethmol	2	99.00 ± 0.434	99.50 ± 0.566 %RSD =0.568 t = 0.68 F = 1.35	100.50 ± 0.519 %RSD =0.516 t = 0.56 F = 1.80	97.50 ± 0.491 %RSD =0.503 t = 0.75 F = 1.80

[$] Recovery amount was the average of five determinants
Tabulated t-value at 95% confidence level is 2.306
Tabulated F-value at 95% confidence level is 6.39

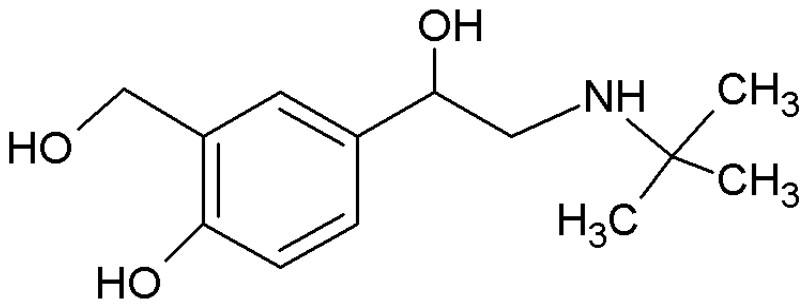


Figure 1: Structure of salbutamol

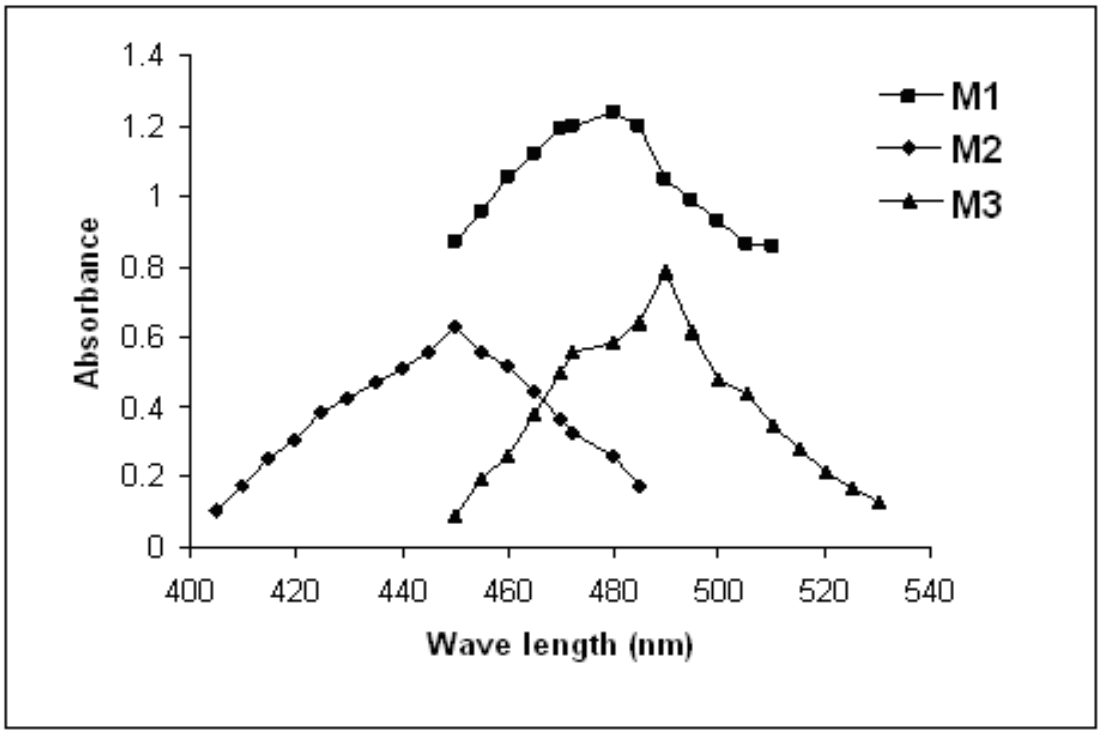
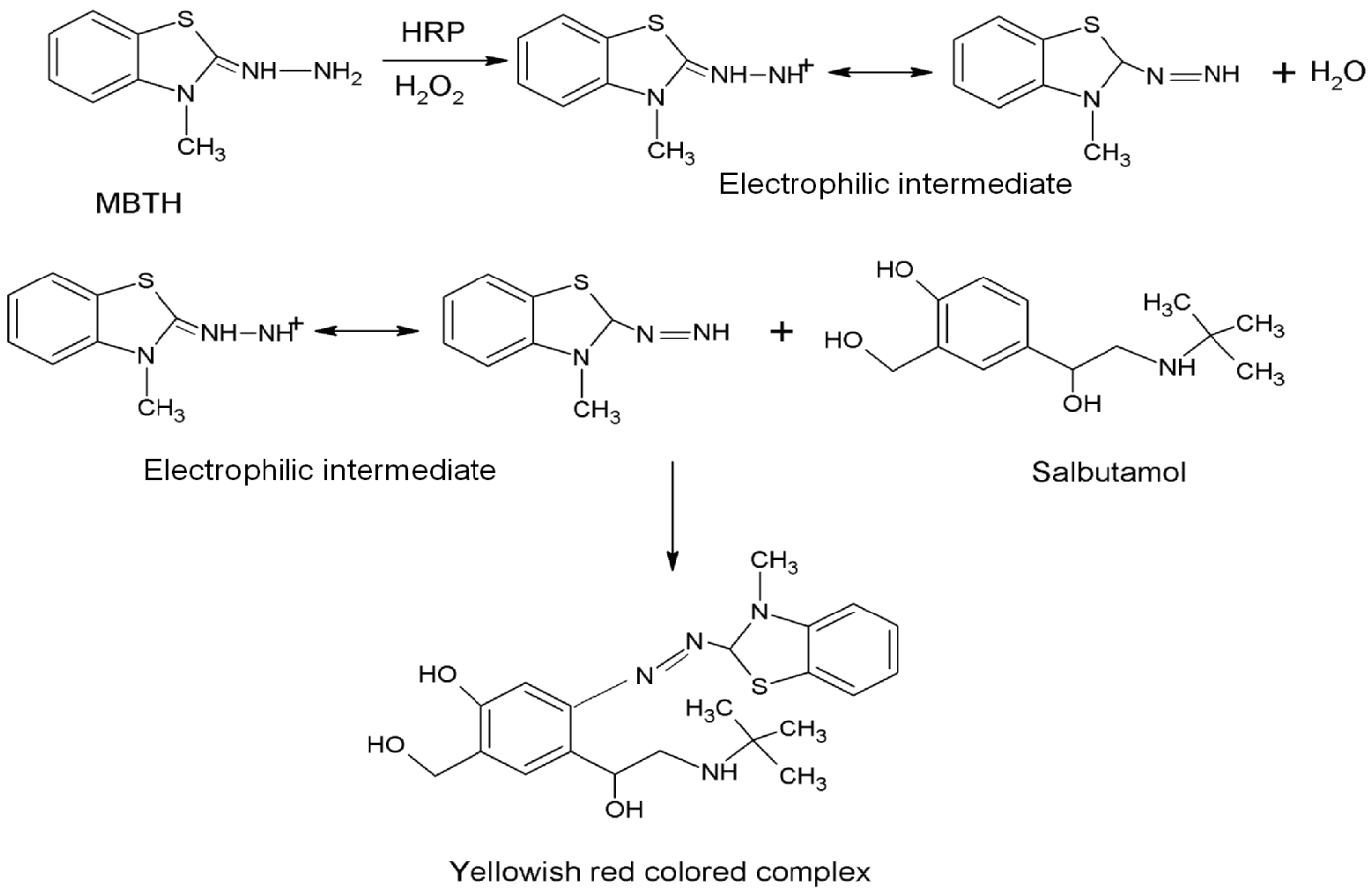
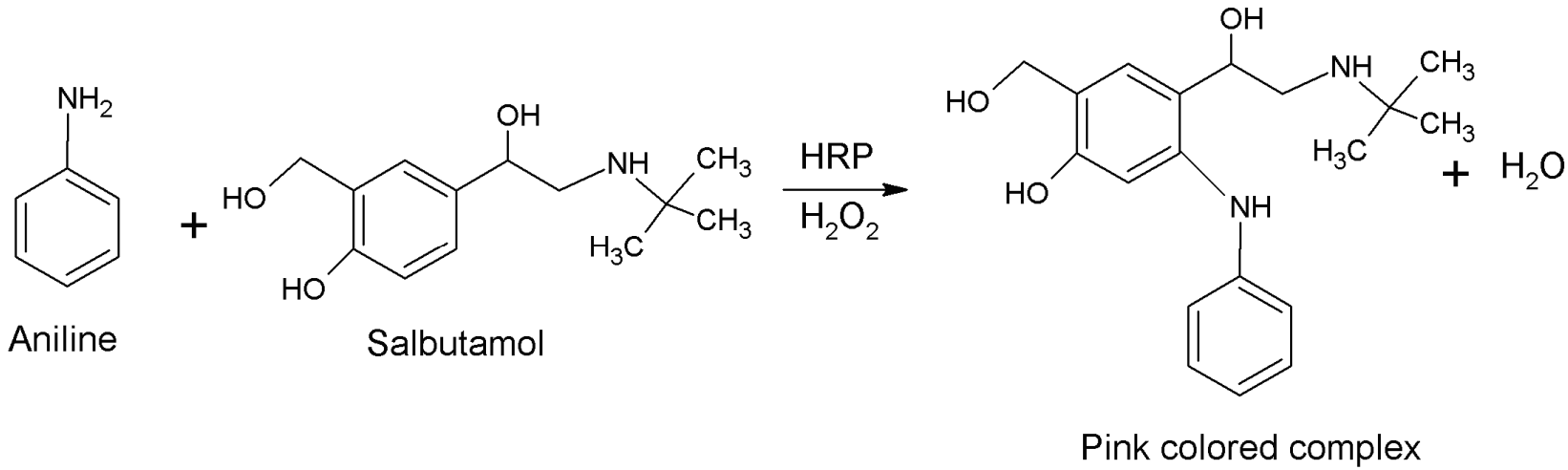


Figure 2: Absorption spectra of SBL-MBTH (M1), SBL-ALN (M2) and SBL-4-AP (M3) complexes

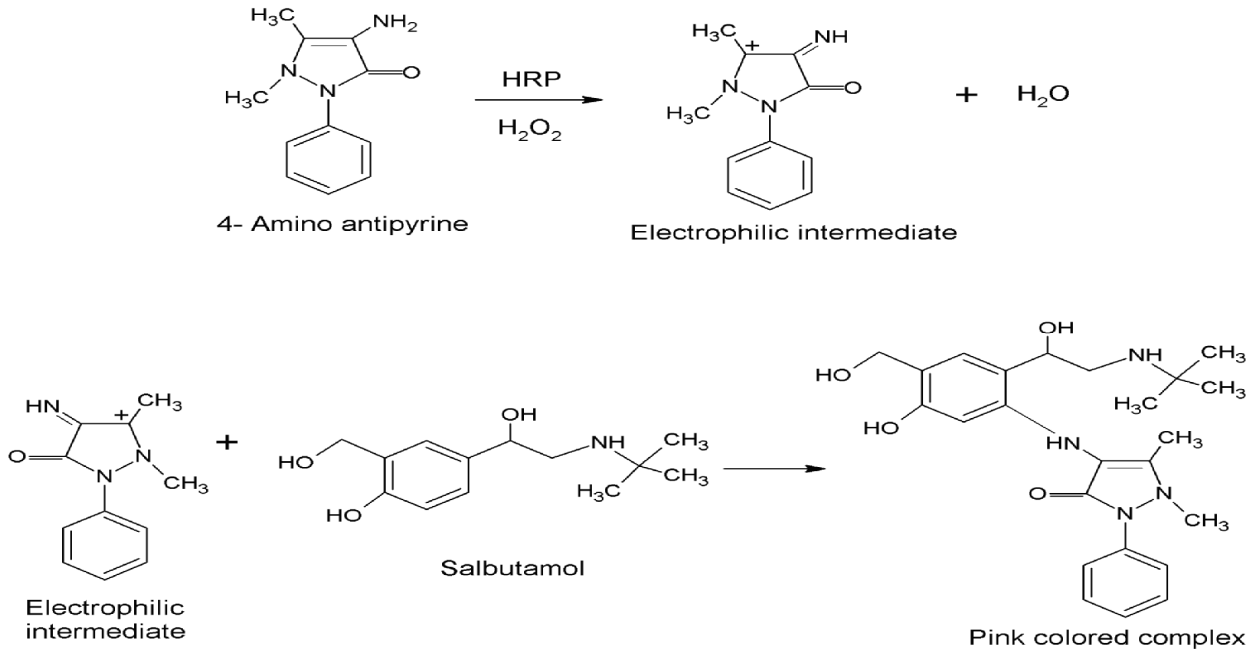
Scheme 1: Proposed mechanism of the reaction between MBTH and Salbutamol



Scheme 2: Proposed mechanism of the reaction between Aniline and Salbutamol



Scheme 3: Proposed mechanism of the reaction between 4 - Amino antipyrine and Salbutamol



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