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Development and Validation of Stability-indicating RP-HPLC method for the simultaneous analysis of Salbutamol, Theophylline and Ambroxol

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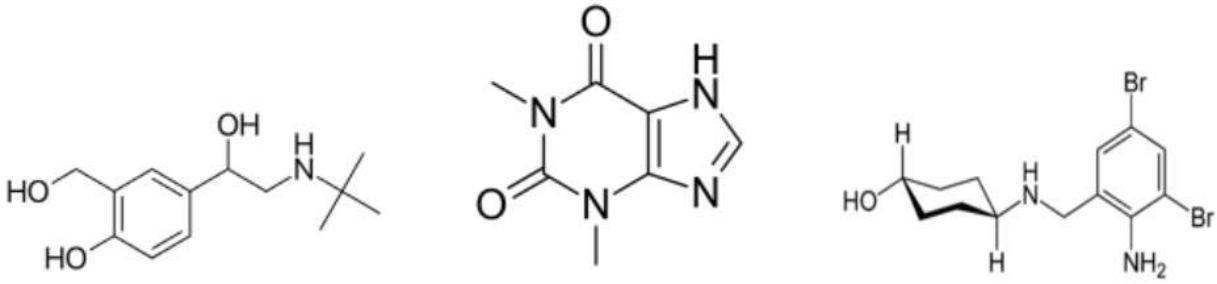
Abstract: In pharmaceutical industry, researchers aim at catering to the need of robust analytical methods for analysis of generic drug products. A simple, novel and efficient stability indicating HPLC method has been developed in a multi component drug formulation for simultaneous estimation of Salbutamol, Theophylline and Ambroxol in presence of their degraded products. This HPLC method uses Inertsil ODS C18 column HPLC column, phosphate buffer pH 6.3 and Methanol: Water: Acetate Buffer 60:35:05 v/v as mobile phase in isocratic mode with UV detection at 239 nm. The method was validated and found to be precise, robust, accurate, linear (range of 1-6µg/ ml, 50-300 µg/ ml and 15-90 µg/ ml for Salbutamol, Theophylline and Ambroxol respectively), and specific for degraded products ensuring suitability of the method for quantitative determination of Salbutamol, Theophylline and Ambroxol. The method is stability-indicating, and therefore qualified and reliable for demonstrating and detecting any expected change or degradation in the drug product during stability studies. The method developed here is found to be novel, robust and rugged enough to reproduce accurate and precise results under different method conditions.

Keywords: Pharmaceutical formulation; HPLC method; Salbutamol; Theophylline; Ambroxol; Validation; Stability indicating method

INTRODUCTION

Salbutamol, a short-acting, selective β 2-adrenergic receptor agonist [1, 2] is typically used to treat or prevent bronchospasm in patients with asthma, bronchitis, emphysema, and other lung diseases [3-4]. It is also known as albutero. It is also be used to treat high blood potassium levels [3].

It is usually used by inhaler or nebulizer. Theophylline, also known as 1, 3 -dimethylxanthine, is a methylxanthine drug used in therapy for respiratory diseases such as Chronic Obstructive Pulmonary Disease (COPD), asthma [5-6] and also for the treatment of the symptoms and reversible airflow obstruction associated with chronic asthma and other chronic lung diseases, such as emphysema and chronic bronchitis. Ambroxol is a secretolytic agent used in the treatment of respiratory diseases associated with viscid or excessive mucus [7-9]. It is the active ingredient of Mucosolvan, Lasolvan or Mucoangin. Ambroxol is indicated as "secretolytic therapy in bronchopulmonary diseases associated with abnormal mucus secretion and impaired mucus transport. It promotes mucus clearance, facilitates expectoration and eases productive cough, allowing patients to breathe freely and deeply" [10-11]. Ambroxol is available as syrup, tablets, pastilles, dry powder sachets, inhalation solution, drops and ampules as well as effervescent tablets.



Salbutamol **Theophylline** **Ambroxol**

Fig-1: Structures of the drugs

In literature survey for the analysis of Salbutamol, Theophylline and Ambroxol confirms that only one analytical method was available for the simultaneous analysis of three drugs in combined dosage forms. Sivasubramanian [12] has developed assay method for the simultaneous estimation of Salbutamol, Theophylline and Ambroxol. Other methods available were the estimation of Salbutamol [13-22], Theophylline [24-26] and Ambroxol [27-39] in single or in combination with other drugs. No stability indicating HPLC method reported for the simultaneous estimation of Salbutamol, Theophylline and Ambroxol. Hence in this study we attempted to develop a novel stability indicating HPLC method for the simultaneous estimation of Salbutamol, Theophylline and Ambroxol in pharmaceutical formulations.

MATERIALS AND METHODS

Instrumentation

To develop a HPLC method for simultaneous estimation of Salbutamol, Theophylline and Ambroxol isocratic PEAK-HPLC instrument equipped with a LC 20AT pump for solvent delivery and variable wavelength programmable LC – 7000 UV-detector is utilised. Inertsil ODS C18 column (250 mm x 4.6 mm, 5µm) was used for chromatographic separation. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234). A 20 µL Rheodyne inject port was used for injecting the samples. Data was analyzed by using PEAK software.

Materials

Analytically pure Salbutamol, Theophylline and Ambroxol were obtained as gift samples from reputed Pharmaceutical companies. Methanol, water (Merck, Mumbai, India) were of HPLC grade, while Acetate Buffer used for the preparation of mobile phase were of analytical grade (Merck Specialties Private Limited, Mumbai, India). Ultipor N_{66} Nylon 6, 6 membrane sample filter paper for the analysis was supplied by Millpores® (Millipores Ltd. Banglore). Formulations of Ambrolite-ST contains a combination of Salbutamol, Theophylline and Ambroxol containing labeled amount of Salbutamol - 2mg, Theophylline – 100mg and Ambroxol - 30mg were procured from local market.

Preparation of standard solution

Standard stock solution of Salbutamol, Theophylline and Ambroxol drug (1mg/ml) was prepared by accurately weighing about 100 mg of each drug in 100 ml volumetric flask separately. Then the drugs were dissolved with 25 ml of methanol, and sonicated to dissolve completely and made up to the mark. The contents were mixed well and filtered through Ultipor N_{66} Nylon 6, 6 membrane sample filter paper. Appropriate volumes of these solutions were further diluted with mobile phase to get required concentrations for construction of calibration curve.

Procedure for pharmaceutical formulation:

Sample solution was prepared by a composite of 20 (Salbutamol, Theophylline and Ambroxol) combination tablets (Ambrolite-ST: 2 mg of Salbutamol and 100 mg of Theophylline and 30 mg Ambroxol) were grinded to fine, uniform size powder. An amount of drug equivalent to 100 mg of Theophylline was accurately weighted and quantitatively transferred into 100 ml volumetric flasks. Approximately 30 ml mobile phase was added and the solution was sonicated for 15 min. Then flask was makeup to volume with mobile phase and mixed well. This solution is filtered through 0.45µm nylon 6, 6 membrane filter paper. Then an amount of the solution was diluted with mobile phase to a concentration of 200 µg/ml of Theophylline. Then based on the label claim of the both the drug in the formulation, a concentration of 4 µg/ml of Salbutamol and 60 µg/ml of Ambroxol solution was obtained.

Method development and chromatographic conditions:

Various trials have been conducted by optimizing the mobile phase, column, detector wavelength etc. in order to achieve the separation of Theophylline, Salbutamol and Ambroxol in mixture solution. Eventually mobile phase consisted of Methanol: Water: Acetate Buffer 60:35:05 v/v at pH 6.3 was selected as optimized solvent for separation. Inertsil ODS C18 column used for separation at UV

detector wavelength 239 nm. An isocratic elution was carried out at 1.0 ml/min of mobile phase flow rate.

Method Validation:

Once optimum separation conditions achieved, method is validated to ensure its suitability and reliability for routine use in estimation of % content of active ingredients in a pharmaceutical formulation. The method was validated according to ICH Q2B guidelines for validation of analytical procedures in order to determine the linearity, sensitivity, precision and accuracy for the analyte. Validation parameters adopted are as follows:

Specificity

The specificity of the developed method is established to prove the absence of interference from placebo peaks (excipients) which is part of the required pharmaceutical preparations. It is also determined by checking blank and standard drugs retention times and identification of the drugs in the sample by comparing the retention times.

Sensitivity

Limit of Detection (LOD) and Limit of Quantification (LOQ) of Theophylline, Salbutamol and Ambroxol is determined using this method by analyzing different dilute solutions of Theophylline, Salbutamol and Ambroxol and measuring signal to noise ratio. The LOD is the concentration that gives a signal to noise ratio of ≥3, while the LOQ in sample can be determined with acceptable precision and accuracy with a signal to noise ratio of ≥10.

Accuracy

The accuracy of the method was based on the percentage recovery of the analyte that was added to weighed amounts at level 50%, 100% and 150% compared to the declared amounts. The results of determinations along with statistical assessment, including the mean, standard deviation, relative standard deviation (RSD %) are presented in the Table 3.

Precision

The precision of the method was confirmed by repeatability and intermediate precision. The repeatability was performed by the analysis of standard solution (4 µg/ml of Salbutamol, 200 µg/ml of Theophylline and 60 µg/ml of Ambroxol) and was repeated for six times with the same concentration. The precision of the method was confirmed by intraday and inter day analysis.

Linearity

Linearity was evaluated by determining six working standard solutions at a concentration range of 1-6 µg/ml for Salbutamol, 50-300µg/ml for Theophylline and 15-90 µg/ml for Ambroxol. Triplicates of such solutions were prepared. Each was analyzed to plot a calibration curve. Slope, intercept and coefficient of determination of the calibration curves were calculated to ascertain linearity of the method.

Ruggedness/Robustness

The ruggedness test is defined as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different labs, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst. In present study, determination of 4µg/ml of Salbutamol, 200 µg/ml of Theophylline and 60 µg/ml of Ambroxol were carried out by different analysts.

Forced degradation studies

To perform the forced degradation study 50 mg each drug is subjected to acidic, alkaline, oxidizing, thermal and photolytic conditions. For acidic degradation the drug was heated under reflux with 0.1 M HCl at 80º C for 2 h and the mixture was neutralized. For alkaline degradation the drug was treated with 0.1 M NaOH at 80º C for 2 h and the mixture was neutralized. For degradation under oxidizing conditions the drug was heated under reflux with (30%, v/v) H_2O_2 at 80º C for 2 h. For thermal degradation the powdered drug was kept at 70º C for 48 h. For photolytic degradation the powdered drug was exposed to sunlight for 48 h. The placebo was also subjected to the same stress conditions to determine whether any peaks arose from the declared excipients. After completion of the treatment, the solutions were left to return to room temperature and diluted with solvent mixture to furnish 30µg/ml solutions. The purity of the drug peak obtained from the stressed sample was measured with UV detector and compared the chromatograms of untreated drugs in tablet solution.

RESULTS AND DISCUSSION

Preliminary studies involved are trying different stationary phases and testing several mobile phase compositions for the effective separation of Salbutamol, Theophylline and Ambroxol. Method development is started with testing three reverse-stationary phases (C4, C8, and C18 columns). These analytes have retention using all these stationary phases, but for good separation of the three analytes and the degradation products, stationary phase C18 (250 mm × 4.0 mm, 5 µm) was found to be the best one for optimum separation, as shown in Figure 2. Regarding the mobile phase, a mixture of Methanol: Water: Acetate Buffer 60:35:05 v/v at pH 6.3 was tested both in isocratic and gradient elution. Isocratic elution is found to be successful for the separation of the three

analytes and the degradation products. Different pH values of the buffers were tested and found that pH 6.3 was the best as it gave a better separation of the three analytes and degradation products. Different flow rates of 1.2, 1.0, and 0.8 ml/min were tested, and observed that 1.0 ml/min was the best one. Room temperature was good for this separation and it was used in the whole separation. Ultraviolet detection at 239 nm was used as it was found to be the optimum wavelength for Salbutamol, Theophylline and Ambroxol, as it gave a high signal-to-noise ratio and a high peak area. Using these conditions, good separation of analytes and degradation products were obtained (Figure 2) as given in Table 1.

An attempt has been made to develop a fast, sensitive, precise, reproducible and economical analytical method for simultaneous estimation of Salbutamol, Theophylline and Ambroxol in their combined dosage form and also to determine the stability indicating activity. System suitability parameters were measured to verify the system, method, and column performance by evaluation of the column efficiency and the ability to separate peaks (Figure 2). Number of theoretical plates, tailing factor, resolution of the chromatogram are reported and found within the acceptable limit and presented in Table 2.

Table-1: Optimized chromatographic conditions

S.NO	Parameter	Results
1	MP	Methanol: Water: Acetate Buffer in the ratio Of 60:35:05 (v/v)
2	Wavelength	239nm
3	Stationary Phase	Inertsil ODS C18 column
4	pH of MP	6.3
5	Flow Rate	1.0ml/min
6	Pump Mode	Isocratic
7	Pump Pressure	11.2±5MPa

Table 2: System suitability Parameters

Parameter	Salbutamol	Theophylline	Ambroxol
API Concentration	4 µg/ml	200 µg/ml	60 µg/ml
RT	3.36 min	7.485 min	8.40 min
Resolution	-	14.8	5.7
Area	45843	443155	104658
Theoretical Plates	12114	14117	77010
Tailing Factor	0.87	0.81	1.02

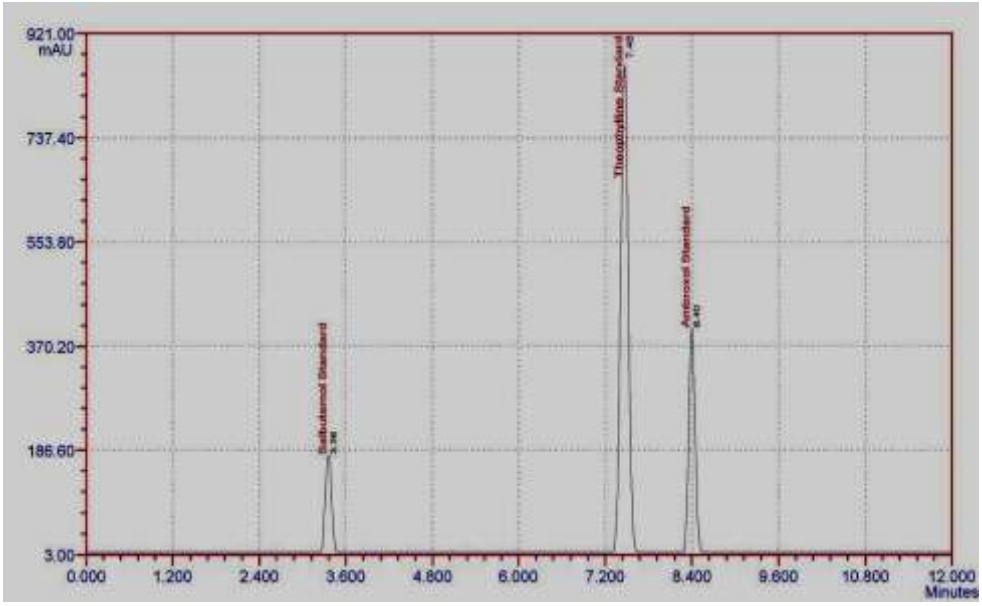


Fig-2: Standard chromatogram of Salbutamol, Theophylline and Ambroxol

In this method, the drugs obey Beer's law in the concentration range of 1-6 µg/ml, 50-300 µg/ ml and 15-90 µg/ ml for Salbutamol, Theophylline and Ambroxol respectively (Figure 3). The regression analysis equation was y = 9068x + 9964 and correlation coefficient (r^2) was 0.999 for salbutamol, y = 1881x + 76956 and correlation coefficient (r^2) was 0.998 for Theophylline and y = 1381x + 20843 and correlation coefficient (r^2) was 0.998 for Ambroxol. A very low values for the statistical parameters like standard deviation of slope, intercept 122.25, 476.9 for Salbutamol, 42.49,8288 for Theophylline and 35.25, 2062.5 for Ambroxol indicate that there is a linear relationship between the concentration and peak area. A very low standard deviation in quantitative analysis results 0.0334 for Salbutamol, 3.39 for Theophylline and 0.955 for Ambroxol indicate that the quantity reported is accurate and acceptable. Hence the quantity of Salbutamol, Theophylline and Ambroxol can be determined accurately using the calibration graphs were represented in Figure 3.

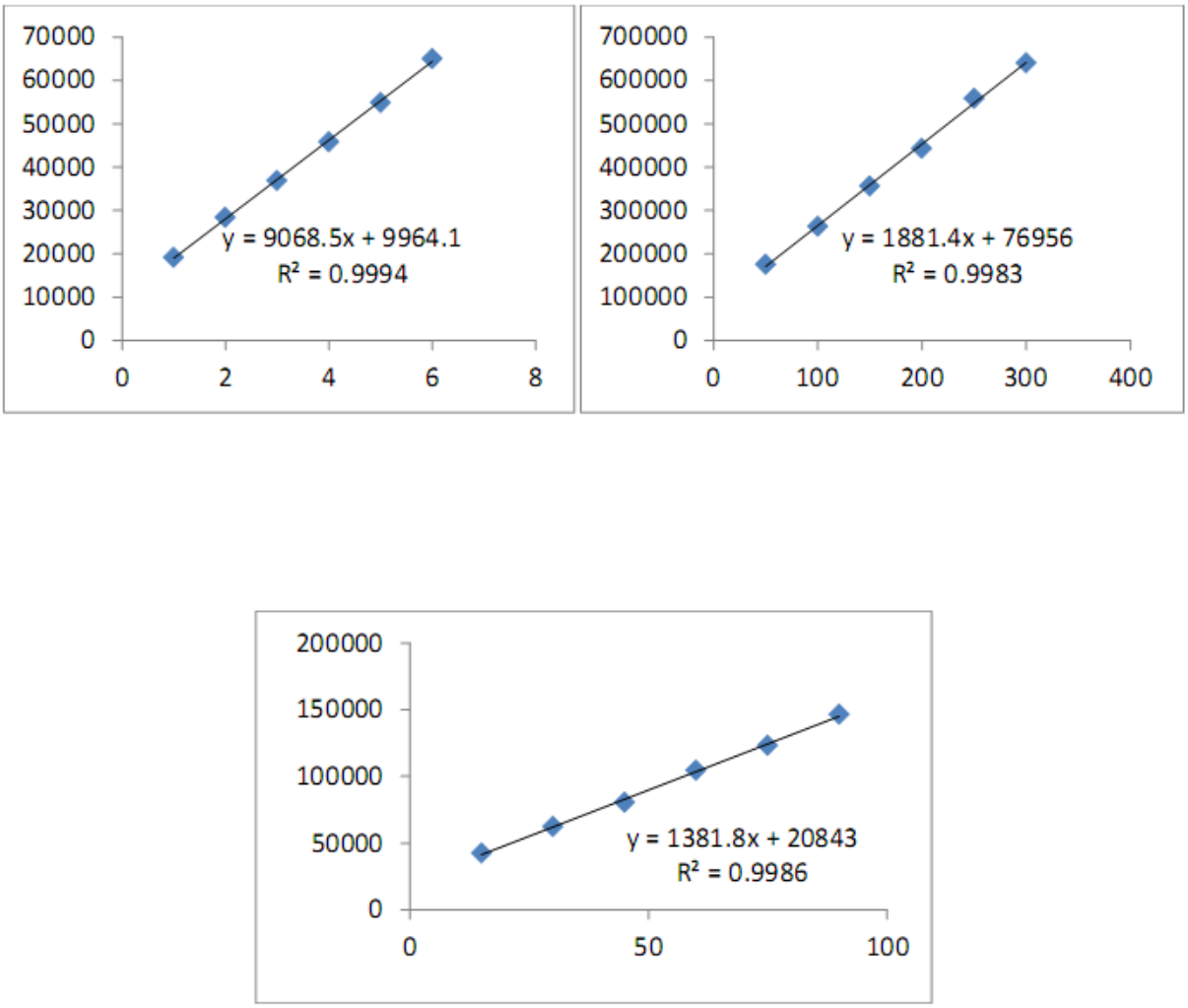


Fig-3: Calibration graph for Salbutamol, Theophylline and Ambroxol (x-axis conc. µg/ml and y-axis peak area)

Precision studies like intraday, inter-day and ruggedness results were found to be within the limit. The values of % RSD of intraday, inter day and ruggedness are found to be 0.79, 0.307 and 0.678 . Mean recovery 99.21%, 99.25% and 98.95% for Salbutamol, 100.29%, 98.63% and 98.05% for Theophylline and 99.29%, 98.96% and 101.09% for Ambroxol reveal that the intraday, inter day precision and ruggedness are in acceptable limit and hence is reliable. The results of recovery studies for tablet were found to be in the range of 98.00 -102.00%. It indicates that there is no interference due to excipients present in the formulation. It can be easily and conveniently adopted for routine quality control analysis. Formulation assay with Ambrolite-ST, commercially available dosage form was found 99.16%, 98.88% and 99.30% for Salbutamol, Theophylline and Ambroxol respectively. It is observed that there is no interference from tablet excipients in these methods (Figure 4). Statistical analysis proves that these methods are repeatable and selective for the analysis of Salbutamol, Theophylline and Ambroxol (Table 3).

Table 3: Summary of validation results

Parameter	Salbutamol	Theophylline	Ambroxol
Intraday precision	0.79	0.948	1.814
Mean Recovery %	99.21	100.29	99.29
Inter-day precision	0.307	1.405	1.770
Mean Recovery %	99.25	98.63	98.96
Ruggedness	0.678	0.973	0.788
Mean Recovery %	98.95	98.05	101.09
Robustness (% of change)	0.523-1.475	0.377-1.986	0.283-1.945
Recovery %	98.534-101.560	98.847-101.097	98.021-101.001
LOQ	0.10 µg/ml	2.0 µg/ml	0.25 µg/ml
LOD	0.03 µg/ml	0.5 µg/ml	0.07 µg/ml
Formulation assay	99.169%	98.885%	99.303%
	F= 1.22	F= 1.654	F= 14.33
	t= 0.12	t= 2.49	t= 2.87

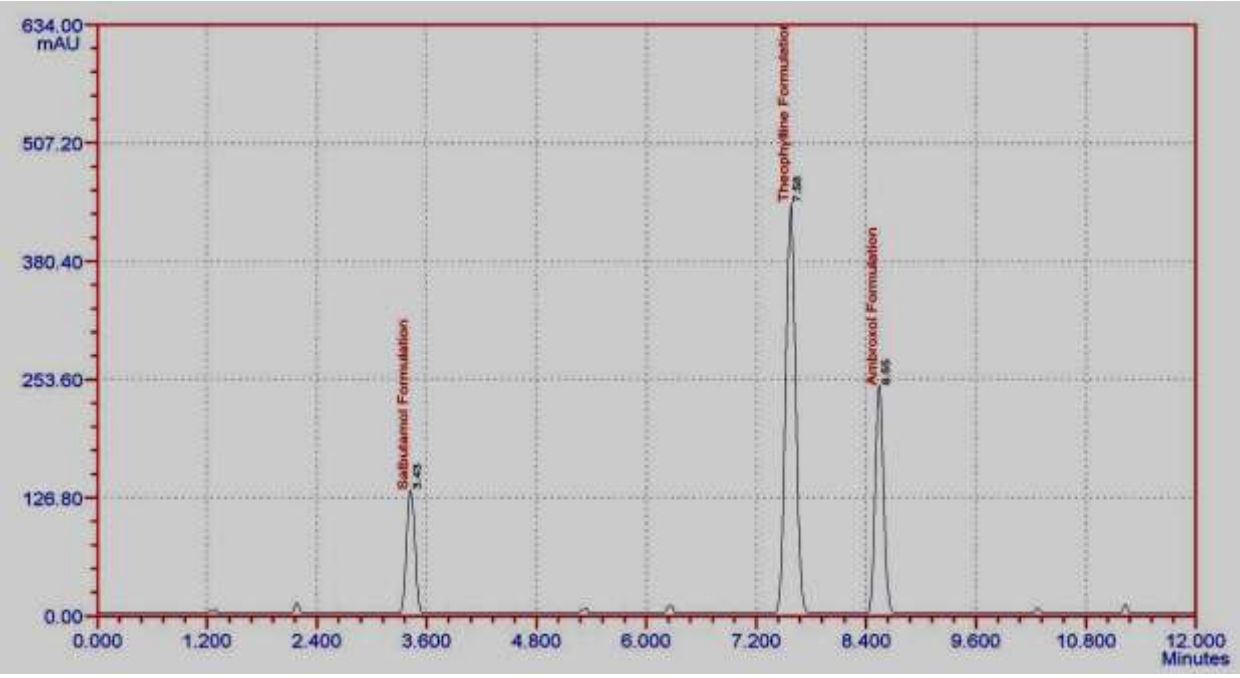
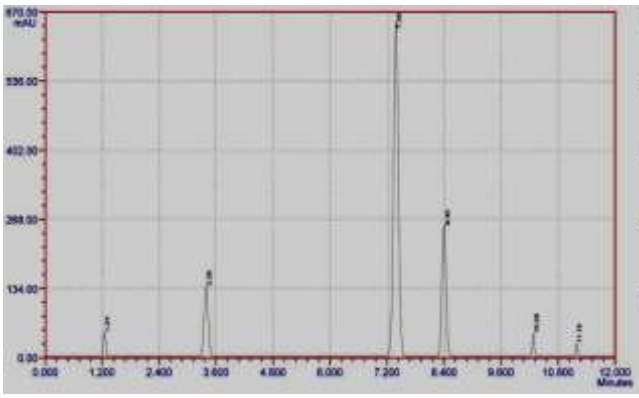


Fig-4: Formulation chromatogram of Salbutamol, Theophylline and Ambroxol

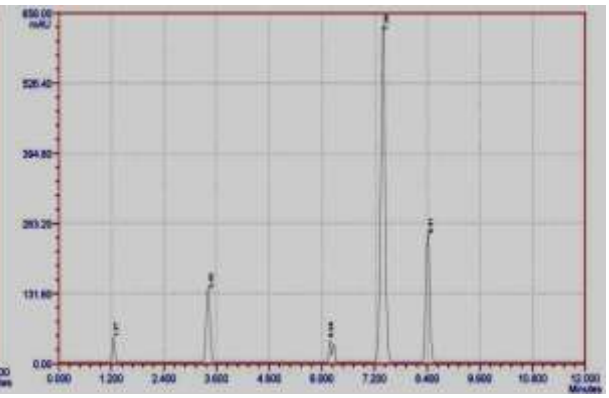
The proposed validated liquid chromatographic method was successfully applied to study the stress degradation property of Salbutamol, Theophylline and Ambroxol. The results of forced degradation studies were given in Table 4 and Figure 5**.** Results are indicating that the method is successfully separated the degradation products and identified separately. The results reveal that drugs are sensitive to the base and peroxide where four additional peaks of degraded products were found. Salbutamol and Theophylline percentage of degradation was found high in peroxide and UV light. Compared to other two drugs Ambroxol was found stable where less percentage of degradation was found in all conditions.

Table 4: Results of Forced Degradation

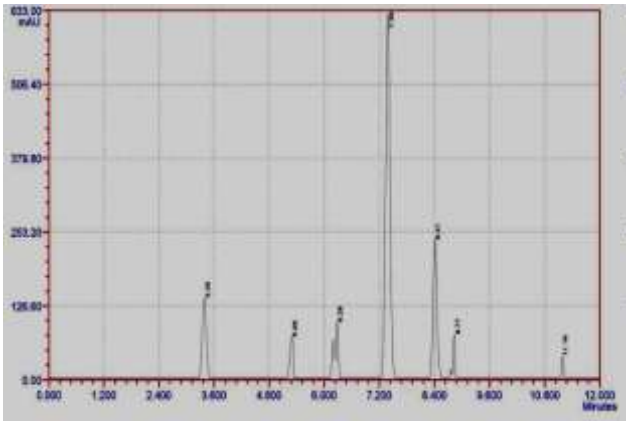
Condition	No of Additional Peaks Observed	Salbutamol Peak Area	Salbutamol % Degradation	Theophylline Peak Area	Theophylline % Degradation	Ambroxol Peak Area	Ambroxol % Degradation
Acidic	3	43042	6.120	412379	6.945	101413	3.101
Aqueous	2	41550	9.364	437872	1.192	100917	3.574
Base	4	40981	10.606	409141	7.675	100115	4.341
Light	3	41457	9.567	413242	6.750	98906	5.496
Peroxide	4	40562	11.520	399136l	9.933	100893	3.597
Thermal	2	44388	3.174	418384	5.590	98719	5.675
UV Light	4	40562	11.520	390394	11.906	100893	3.597



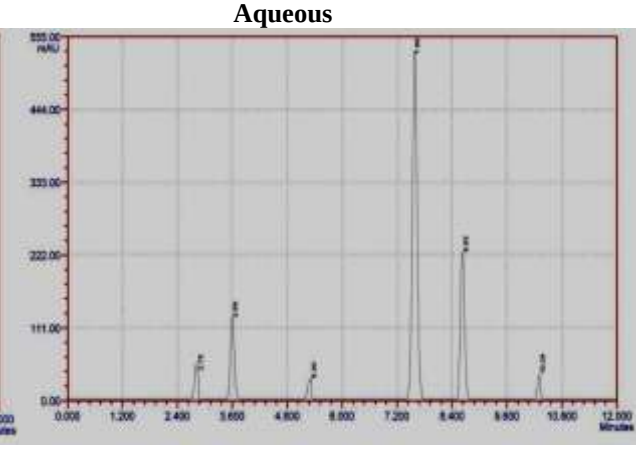
Acid



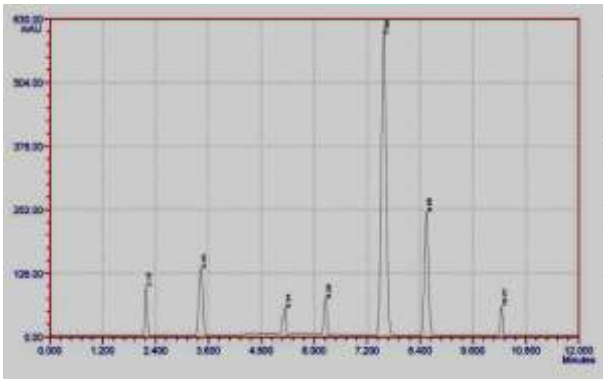
Aqueous



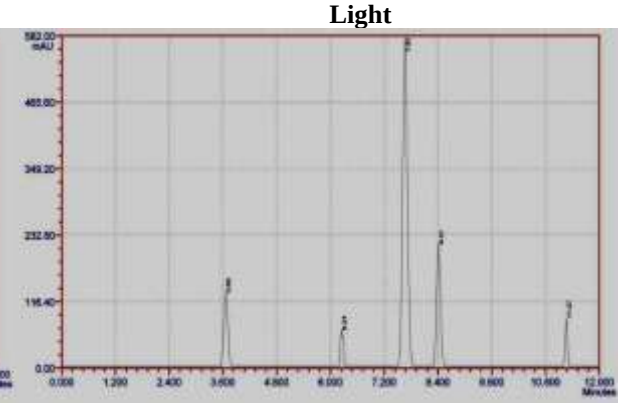
Base



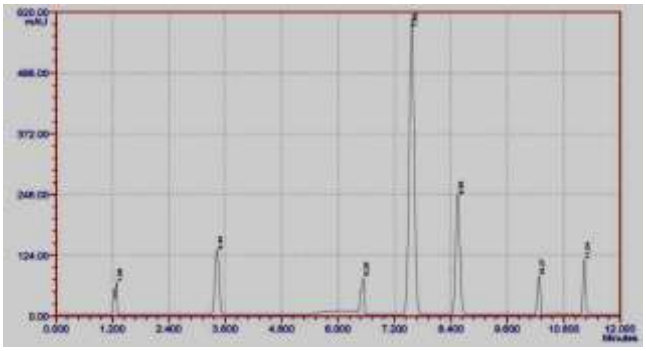
Light



Peroxide



Thermal



UV Light

Fig-5: Chromatograms of force degradation studies

CONCLUSIONS

A simple, accurate, and precise stability-indicating HPLC method was developed and validated for routine qualitative and quantitative analysis of Salbutamol, Theophylline and Ambroxol respectively in a tablet formulation. The method is stability-indicating, and therefore qualified and reliable for demonstrating and detecting any expected change or degradation in the drug product during stability studies. As there is no literature available for the stability indicating HPLC method the simultaneous estimation of Salbutamol, Theophylline and Ambroxol, the method developed here was found to be novel, robust and rugged enough to reproduce accurate and precise results under different method conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. This work and this article do not contain any studies with animals or human participants performed by any of the authors.

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