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Development of a Validated HPLC Method for Simultaneous Estimation of Multidrug Therapy

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Abstract

Salbutamol, theophylline, and ambroxol may all be measured simultaneously in pharmaceutical formulations using a straightforward, accurate, and exact reverse phase high performance liquid chromatographic (RP-HPLC) approach that has been developed and validated. On an Inertsil ODS-3V (250 × 4.6 mm, 5µ) column, the chromatographic separation was accomplished with a mobile phase that included acetonitrile and phosphate buffer (pH 3.0) at a 55:45 v/v ratio at a flow rate of 1 mL/min. At 225 nm, detection was done. Salbutamol, theophylline, and ambroxol were shown to have retention periods of 2.317, 3.808, and 5.863 minutes, respectively. Salbutamol, Theophylline, and Ambroxol were shown to be linear in the concentration range of 0.5-3.0 µg/mL, 25-150 µg/mL, and 7.5-45 µg/mL, respectively, with correlation coefficients more than 0.999 when the suggested approach was verified in accordance with ICH recommendations. It was discovered that the devised procedure was robust, specific, accurate, and exact. The new HPLC method's capacity to indicate stability was shown by forced degradation trials. Salbutamol, theophylline, and ambroxol in pharmaceutical dose forms may all be routinely analyzed using the described approach.

Keywords: Ambroxol, theophylline, HPLC, salbutamol, simultaneous estimation, validation, and tablet dosage form

1. Introduction

As a beta-2 adrenergic receptor agonist, salbutamol (SAL), also known chemically as 4-[2-(tert-Butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol [1], is used as a bronchodilator to treat asthma and chronic obstructive pulmonary disease (COPD). Theophylline (THE), also known as 1,3-dimethyl-2,3,6,7-tetrahydro-1H-purine-2,6-dione, is a methylxanthine medication that relaxes the smooth muscles in the lungs' airways to provide a bronchodilator effect. In diseases including asthma and COPD, it is used to relieve bronchoconstriction [2]. The expectorant medication ambroxol (AMB), also known chemically as trans-4-(2-Amino-3,5-dibrombenzylamino)-cyclohexanol, is used to treat respiratory conditions linked to sticky or viscous mucus [3]. It lessens coughing and removes mucus from the respiratory system.

When combined, the effects of SAL, THE, and AMB are synergistic. AMB lowers the viscosity of respiratory secretions and makes them easier to clear, THE improves mucociliary clearance and relaxes bronchial smooth muscles, and SAL functions as a bronchodilator to relax airway smooth muscles. In illnesses like asthma and COPD, a fixed dosage combination of these three medications helps to effectively relieve symptoms including bronchospasm, dyspnea, and excessive mucus production [3]. Numerous analytical techniques, such as UV spectrophotometry and high performance liquid chromatography (HPLC), have been described for the quantification of these medicines either alone or in combination [4]–[6]. For quality control analysis of fixed dosage combination products, a simultaneous estimate approach for all three medications in a single run is not yet available.

The current research was aimed to develop a simple, accurate, precise and rapid reverse phase HPLC method for the simultaneous estimation of SAL, THE and AMB in pharmaceutical formulations. The developed method was thoroughly validated as per ICH guidelines and applied for analysis of marketed fixed dose combination tablets containing these three drugs. Forced degradation studies were also performed to demonstrate the stability-indicating ability of the method.

2. Material and methods

2.1. Materials

Pharmaceutical grade standards of SAL, THE and AMB were received as generous gifts from Spectrum Pharma Research Labs, Hyderabad. Tablet formulation containing 2 mg SAL, 100 mg THE and 30 mg AMB (Ambrolite-ST) was procured from a local pharmacy. HPLC grade acetonitrile and analytical grade potassium dihydrogen phosphate were used to prepare the mobile phase. Milli-Q water was used throughout the analysis.

2.2. Instrumentation

The HPLC system comprised of a Waters alliance model 2695 separation module with 2996 PDA detector. Data was acquired and processed using Empower-2 software. Analysis was performed on a Inertsil ODS-3V (250 x 4.6 mm, 5µ) column

2.3. Method development

2.3.1. Conditions of the Chromatography
The mobile phase was supplied at a flow rate of 1.0 mL/min and included phosphate buffer (pH 3): acetonitrile at a ratio of 55:45 v/v. Before use, the mobile phase was degassed in an ultrasonicator and filtered through a 0.45µ membrane filter [7]. At room temperature, isocratic elution was carried out using a 225 nm detection wavelength. Ten microliters was the injection volume.

2.3.2. Preparing Standards and Samples
SAL (20 µg/mL), THE (1000 µg/mL), and AMB (300 µg/mL) standard stock solutions were made by dissolving the required quantities of each medication in the appropriate solvent. The stock solutions were diluted to provide working standards in the concentration range of 0.5-3.0 µg/mL for SAL, 25-150 µg/mL for THE, and 7.5-45 µg/mL for AMB. To create the sample stock solution, tablet powder equal to the average tablet weight was removed and appropriately diluted. To get the sample solution, further dilutions were done [8].

2.3.3. Preparation of Standards and Samples
required quantities of each medication were dissolved in a minimum volume of methanol, sonicated for 30 minutes, and diluted to the required level with mobile phase to create standard stock solutions of SAL (20 µg/mL), THE (1000 µg/mL), and AMB (300 µg/mL) [9].

By diluting the stock solutions with mobile phase, working standards with 0.5-3.0 µg/mL of SAL, 25-150 µg/mL of THE, and 7.5-45 µg/mL of AMB were created.

To prepare the sample, one tablet's worth of tablet powder was weighed, extracted in 100 milliliters of methanol, and then shaken for 30 minutes, sonicated for 20 minutes, and centrifuged for 10 minutes at 5000 rpm. To create a sample stock solution with equal concentrations of 20 µg/mL SAL, 1000 µg/mL THE, and 300 µg/mL AMB, the supernatant was collected and appropriately diluted.

2.3.4. Adequacy of the System

By infusing a working standard solution and examining system performance metrics such as theoretical plates, tailing factor, and retention time, system appropriateness was assessed. They used criteria such as resolution between drug peaks > 2 and %RSD of retention time < 2 [10].

2.4. Method Validation

2.4.1. Linearity and Range

Linearity of the method was evaluated by preparing calibration standards of SAL (0.5-3 µg/mL), THE (25-150 µg/mL) and AMB (7.5-45 µg/mL) in triplicate. The peak area vs concentration data was treated by linear least square regression analysis. Calibration curves were constructed by plotting average peak area versus concentration of each analyte. The coefficient of regression (r^2) was calculated for each compound [11].

2.4.2. Precision

The precision of the method was established by carrying out intraday and interday analysis of quality control samples. For intraday precision, QC samples at three different concentration levels were analyzed in triplicate on the same day. Interday precision was checked by analyzing QC samples on three different days over a period of one week. The %RSD of three concentrations was calculated for both intraday and interday studies [12].

2.4.3. Accuracy

The accuracy of the method was determined by spiking extra standards of known concentrations into pre-analyzed sample solutions at three levels (50, 100 and 150% of target QC concentration). The percentage recoveries and %RSD at each level were calculated [13].

2.4.4. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample solutions. The purity of peaks of all three analytes were checked by measuring peak purity testing using PDA detector in the developed method [14], [15].

2.4.5. Robustness

To evaluate robustness of the developed method, experimental conditions were deliberately changed and their influence was observed on various method parameters like retention time, tailing factor and theoretical plates. The flow rate of mobile phase was changed by 0.1 mL/min from 1.0 mL/min. Percent organic composition in the mobile phase was varied ±2%. Effect of pH on resolution was also studied [16].

2.4.6. Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on standard deviation of the response and slope of the calibration curve at levels approximating to the LOD and LOQ as per ICH guidelines [10].

2.4.7. Forced Degradation Studies

Forced degradation was performed under various ICH recommended conditions like acid/base hydrolysis, oxidation, photolysis and thermal stress [10]. Degradation samples were analyzed at specified time points and areas of peaks for drugs and degradation products were monitored.

3. Results and discussion

3.1. Optimized chromatographic conditions

The RP-HPLC method for simultaneous determination of Salbutamol, Theophylline, and Ambroxol underwent systematic optimization. Various parameters, including columns, buffers, buffer pH, organic phase composition, organic modifiers, and flow rates, were individually optimized. The final method employed a mobile phase consisting of a 55:45 ratio of Phosphate buffer to Acetonitrile, delivered at a flow rate of 1.0 mL/min, with a 10-minute run time. Equilibration of the column with the mobile phase was performed for 30 minutes prior to sample injection, and detection occurred at a wavelength of 225 nm. The optimized parameters are shown in Table 1.

Table 1 Optimized chromatographic conditions of the proposed method

Parameter	**Description**
Column	An Inertsi10DS— 3V column (250 x 4.6 mm i_d; particle size - 5 micron)
Mobile Phase and Mode	Phosphate buffer. Acetonitrile in the ratio of 55:45 v/v %in a isocratic mode
Diluent	Drugs were dissolved in methanol and the volume was made up with the phosphate buffer solution
Flow Rate	1 ml/min
Run time	11 min
Injection Volume	10 µl
Column temperature	30^{0}C
Detection & Wavelength	2996 PDA-detector and 225 nm
Retention time	2.317 min for Salbutamol, 3.808min for Theophylline and 5.863min for Ambroxol

3.2. Chromatograms and Method Validity

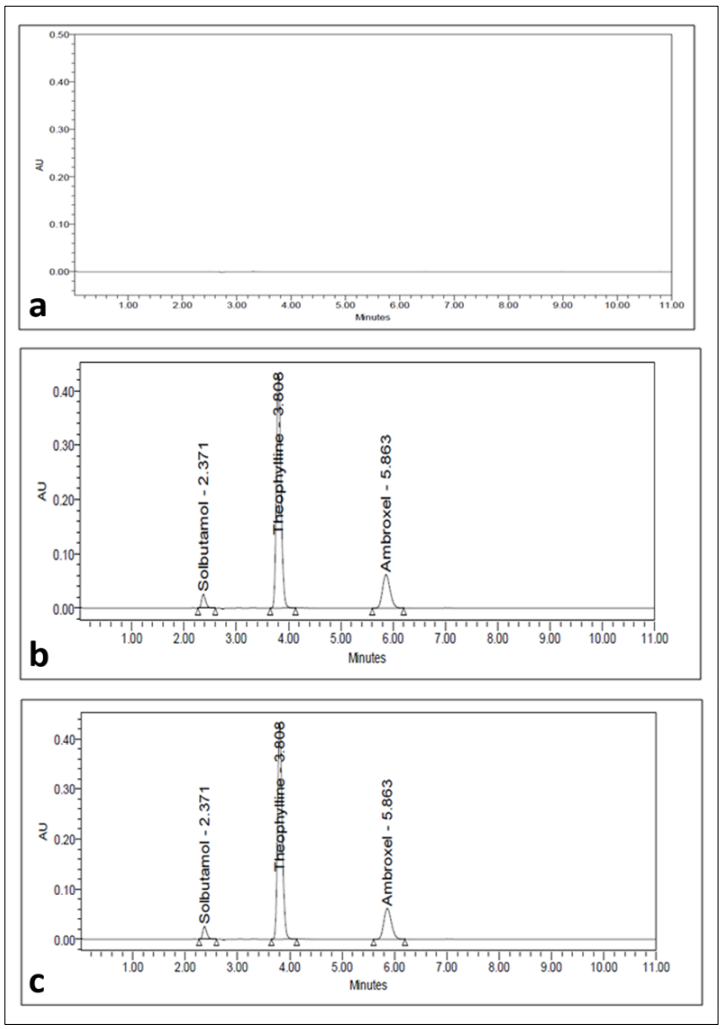

Figure 1 a Chromatogram of blank solution b. Chromatogram of the working standard solution, c. Chromatogram of the drug product

Figure 1a shows the chromatogram of blank solution while 1b displays the chromatogram of the working standard solution, and Figure 1c illustrates the chromatogram of the drug product. The isocratic HPLC method, with an 11-minute run time, effectively separated the three compounds. Blank samples confirmed no interference from excipients. The reproducibility and short run time make this method suitable for routine analysis of pharmaceutical products [8].

3.3. Assay of Formulation

To assess the % purity of the pharmaceutical formulation, 10 µL of working standard and sample solutions were individually injected and analyzed under optimized chromatographic conditions. The % assay results are summarized in Table 2.

Table 2 Results of Assay Ambrolite-ST

Drug	Salbutamol	Theophylline	Ambroxol
Label claim (mg)	2	100	30
Amount found (mg)	1.99	100.19	29.90
% Assay	99.61	100.19	99.67
Limits	98-102%		

3.4. System Suitability

System performance parameters like theoretical plates, tailing factor and retention time for standard mixtures were evaluated as per USP guidelines and are presented in Table 3. All values were within acceptable limits indicating suitability of the system for intended analysis.

Table 3 System suitability test parameters of the proposed method

S. No	Parameters	Salbutamol	Theophylline	Ambroxol	Limits
1	Relative retention time (min)	2.57	4.15	6.32	--
2	% RSD of Retention Time	0.42	0.23	0.175	Not more than 2
3	Peak Area	149635.2	2882163	670507	--
4	% RSD of Peak area	0.30	0.34	0.25	Not more than 2
5	Theoretical plates	4505	9588	8271	More than 2000
6	Tailing factor	1.29	1.16	1.15	Less than 2
7	Resolution	-	> 2	>2	More than 2

3.5. Method Validation

3.5.1. Linearity and Range

Calibration curves were plotted over the concentration range of 0.5-3.0 µg/mL for SAL, 25-150 µg/mL for THE and 7.5- 45 µg/mL for AMB. The correlation coefficients were found to be >0.999 indicating excellent linearity as shown in Figure 2 and Tables 4.

Table 4 Linearity results of salbutamol, theophylline and ambroxol

S.No	Conc. of Salbutamol in µg/mL	Salbutamol area	Conc. of Theophylline in µg/mL	Theophylline area	Conc. of Ambroxol in µg/mL	Ambroxol Area
1	0.5	33661	25	723116	7.5	155685
2	1	72611	50	1356285	15	315833
3	1.5	108892	75	2103704	22.5	484073
4	2	141279	100	2700836	30	630290
5	2.5	173083	125	3478029	37.5	779708
6	3	210977	150	4146869	45	937413
Linearity Range		0.5-3 µg/mL	25-150 µg mL		7.5-45 µg/mL	
Slope		70031	27564		20832	
Intercept		738.7	5371		3135	
r^2		0.999	0.999		0.999	

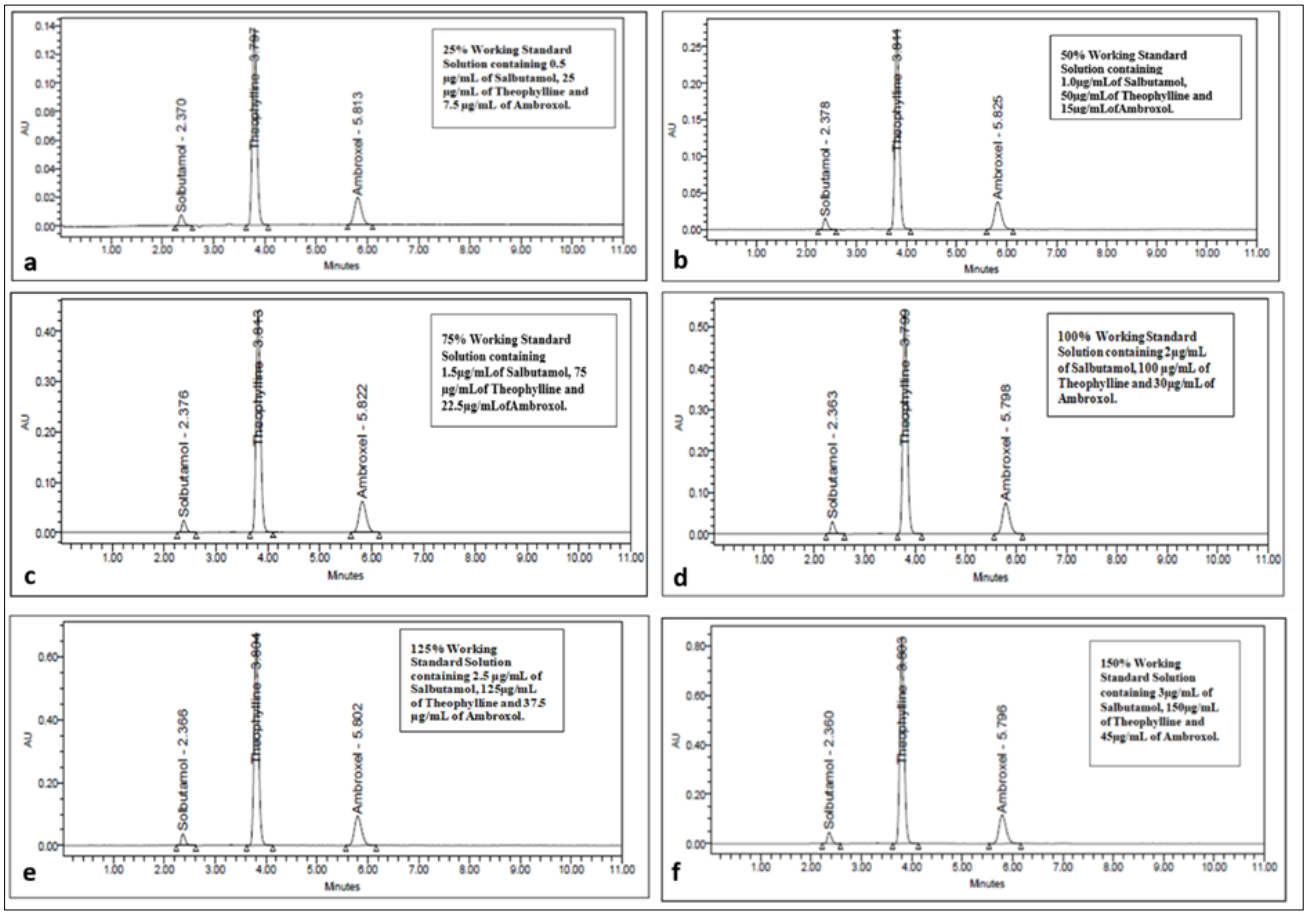

Figure 3 Chromatograms to report the Linearity of Salbutamol, Theophylline and Ambroxol at a. 25%, b. 50% c. 75% d. 100% e. 125% f. 150% working standard solutions

3.6. Precision

Repeatability of sample application and measurement of peak area were evaluated using 6 replicates of sample solution.
%RSD values for retention time and area were within 2% demonstrating excellent precision of the method as shown in Tables 5 and 6.

Table 5 Precision of the developed method using working standard solution

Injection	Salbutamol		Theophylline		Ambroxol	
	Retention Time in min	Peak Area	Retention Time in min	Peak Area	Retention Time in min	Peak Area
1	2.569	149829	4.146	2879298	6.313	670890
2	2.57	149344	4 146	2863773	6.315	669669
3	2.57	149089	4 147	2883619	6.324	667484
4	2.584	149498	4 158	2886673	6.327	670983
5	2.585	149627	4.161	2890447	6.334	671625
6	2.596	150424	4.169	2889170	6.342	672391
Mean	2.579	149635.2	4.1545	2882163	6.325833	670507
Std. dev	0.01	460.82	0	9864.91	0.01	1732.82
%RSD	0.42	0.30	0.23	0.34	0.17	0.25

Table 6 Intraday Precision of working sample solution (Ambrolite-ST)

Sample Preparations	%Assay		
	Salbutamol	Theophylline	Ambroxol
Sample-1	98.42	100.48	99.99
Sample-2	98.78	99.03	101.23
Sample-3	100.23	98.42	100.75
Sample-4	100.72	100.63	99.33
Sample-5	100.09	99.59	100.14
Sample-6	99.8	99.49	99.67
AVG	99.67	99.61	100.19
S.D	0.89	0.845	0.70
%RSD	0.89	0.85	0.70

3.7. Accuracy

Accuracy was determined by calculating mean recovery of the drugs at three concentration levels (50%, 100% and 150%). Average recovery was found in the range of 98-102% indicating high accuracy of proposed method as presented in Table 7.

Table 7 Recovery studies of Salbutamol, Theophylline and Ambroxlin tablet dosage form

% Conc	Salbutamol			Theophylline			Ambroxol		
	Amount Added (µg/ml)	Amount f ound (µg/ml)	% Reco-very	Amount Added (µg/ml)	Amount found (µglntl)	% Recovery	Amount Added (µg/ml)	Amount found (µg/ml)	% Recovery
50	I	0.98	98.77	50	49.47	98.96	15	15.12	100.86
50	I	0.99	99.29	50	50.56	101.13	15	14.87	99.19
50	1	1.00	100.85	50	49.80	99.62	15	15.12	100.87
100	2	1.98	99.26	100	99.00	99.01	30	30.25	100.84
100	2	2.01	100.77	100	100.53	100.53	30	29.85	99.50
100	2	2.00	100.28	100	101.55	101.56	30	29.78	99.27
150	3	2.98	99.41	150	149.49	99.66	45	45.25	100.57
150	3	2.96	98.69	150	150.83	100.56	45	45.40	100.90
150	3	3.03	101.14	150	151.47	100.98	45	44.42	98.73
Average	99.83			99.83			100.08		
SD	0.941			0.941			0.890		
RSD	0.939			0.939			0.889		

3.8. Specificity

Specificity of the method was ascertained by good separation of all three drugs from placebo peaks. No interference of excipients was observed in chromatograms of sample solution indicating specific nature of the method.

3.9. Robustness

Method robustness was demonstrated by deliberately changing chromatographic conditions like flow rate (±0.1 mL/min), organic phase composition (±2%) and detection wavelength (±2 nm). No marked changes in system suitability parameters were observed as shown in Tables 8-10 indicating robustness of method.

Table 8 Results of robustness by variations in flow rate, column temperature, Mobile phase composition of Salbutamol

Sl. No.	Parameter	Used	Peak Area	Retention Time (min)	Plate count	Tailing Factor
Optimized Conditions		1.0 mL/min; 30^{0}C; Phosphate buffer (pH 3): Acetonitrile (55:45)	138673	2.362	4172	1.28
1	Flow Rate (±0.l ml/min)	0.9 mLlmin	189291	3.216	3525	1.34
		I.I DJL:min	148406	2.368	3785	1.31
2	Column Temperarure (±5 ^{0}C)	25^{0}C	153483	2.438	3149	1.31
		35 ^{0}C	149635	2.569	4386	1.29
3	Mobile phase composition	50:50	172489	2.584	4410	1.29
		60:40	157556	2.573	2599	1.27

Table 9 Results of robustness by variations in flow rate, column temperature, Mobile phase composition of Theophylline

Sl No	Parameter	Used	Peak Area	Retention Time	Plate count	Tailing Factor
Optimized Conditions		1.0 mLJmin; 30°c;Phosphate buffer (pH 3): Acetonitrile (55:45)	2662176	3.797	6569	1.16
1	Flow Rate (±0.l ml/min)	0.9 mL/min	3556245	6.344	9571	1.18
		1.1 mL/min	2825334	3.878	8204	1.l6
2	Column Temperature (±5°C)	25°C	2952204	4.092	7850	1.17
		35°C	2889170	4.4 16	9720	1.15
3	Mobile phase composition	50:50	341·6914	4.228	8429	1.17
		60:40	3076379	4.282	8647	1.16

Table 10 Results of robustness by variations in flow rate, column temperature, Mobile phase composition of Ambroxol

Sl No	Parameter	Used	Peak Area	Retention Time	Plate count	Tailing Factor
Optimized Conditions		1.0 Ml/min; 30°c;Phosphate buffer (pH 3): Acetonitrile (55:45)	620548	5.825	7704	1.16
1	Flow Rate (±0.l ml/min)	0.9 mL/min	84645	8.295	8519	1.16
		1.1 mL/min	660354	6.238	6612	1.17
2	Column Temperature (±5°C)	25°C	684563	6.566	7969	1.15
		35 °C	670507	6.313	8284	1.15
3	Mobile phase composition	50:50	769260	6.480	7382	1.15
		60:40	693555	7.242	7576	1.15

3.10. Limit of Detection and Quantitation

The values for the Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined based on the standard deviation of the intercept and slope of the calibration curves. These values demonstrate adequately low limits, ensuring precise and reliable detection and quantitation of the analyzed compounds. Specifically, the LOD values were found to be 0.021 µg/ml for Salbutamol, 1.181 µg/ml for Theophylline, and 0.274 µg/ml for Ambroxol. The corresponding LOQ values were 0.065 µg/ml, 3.578 µg/ml, and 0.8318 µg/ml for Salbutamol, Theophylline, and Ambroxol, respectively.

3.11. Forced Degradation Studies

Drugs were subjected to stress conditions of hydrolysis, oxidation, photolysis, and thermal degradation in order to demonstrate the suggested method's stability indicating ability. As shown in Figure 4, the approach can measure pharmaceuticals in the presence of degradation products since medications were consistently well-resolved from degradation products. Salbutamol's controlled sample showed a high assay of 99.61% with no discernible degradation or purity problems. Degradations using acid, base, peroxide, heat, UV, and water were carried out; the assay and degradation percentages varied for each of these. Interestingly, purity flagging was not activated by any of the criteria.

Theophylline underwent comparable forced degradation investigations; the findings are shown in Table 3.19. The controlled sample showed a 100.19% test with no problems related to purity or deterioration. Degradation tests using acid, base, peroxide, heat, UV, and water revealed differences in assay and degradation percentages. Crucially, no degradation scenario resulted in purity dropping, demonstrating the drug's resilience to these stressful situations.

The controlled sample of ambroxol demonstrated a high assay of 99.67%, with no significant degradation or purity concerns. Acid, base, peroxide, thermal, UV, and water degradations were performed, showing variations in assay and degradation percentages. Notably, none of the conditions resulted in a purity flag, underscoring the stability of Ambroxol under the evaluated stress conditions.

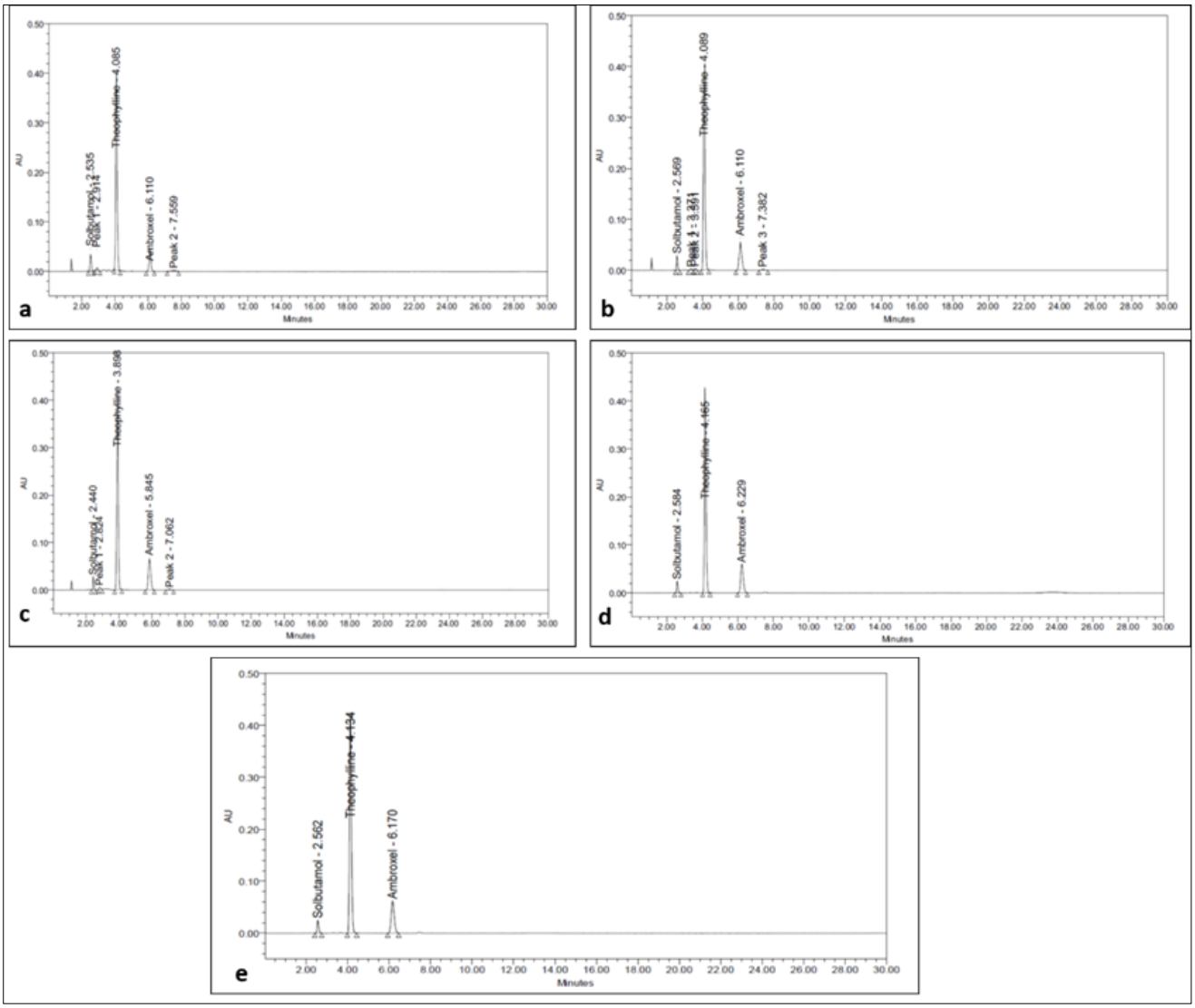

Figure 4 Chromatograms of forced degradation studies a. Peroxide Degradation b. Acid Degradation S tudies c. Base Degradation d. Thermal Degradation e. UV Degradation

4. Conclusion

A simple, precise, accurate and rapid RP-HPLC method was developed and validated for simultaneous analysis of SAL, THE and AMB in tablet dosage form without any interference from excipients. The method was fully validated as per ICH guidelines and can be employed for routine quality control analysis of pharmaceutical formulations containing these drugs.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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