CHEMOMETRICS ASSISTED STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF ACETYL CYSTEINE AND AMBROXOL HYDROCHLORIDE IN BULK AND DOSAGE FORM

Potluri H.[a]*

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ABSTRACT

A simple and precise stability indicating RP-HPLC method was developed for the simultaneous estimation of acetyl cysteine and ambroxol hydrochloride in bulk and dosage form. Experimental design based evaluation using a 23 full factorial design was applied to evaluate coefficient, ANOVA for the establishment of robustness nature of the method. Kromosil (250 mm x 4.6 mm, 5 µ) C18 column at 274 nm of UV detection was used. A composition of 0.1 % ortho phosphoric acid and acetonitrile in the ratio of 28:72 (V:V) was used as the mobile phase with a flow rate of 1.0 mL min^{-1}. Linearity was established over the concentration range of 50-300 µg. mL^{-1} for acetyl cysteine and 7.5-45 µg mL^{-1} for ambroxol hydrochloride. The proposed method was validated and was successfully utilized for the quantitative analysis of tablet formulations containing acetyl cysteine and ambroxol hydrochloride.

Key words: Acetyl cysteine, ambroxol hydrochloride, HPLC, validation, experimental design.

INTRODUCTION

Acetylcysteine (ACE) is an effective mucolytic agent used in the treatment of chronic bronchitis, pneumonia, cystic fibrosis and also to prevent liver damage due to alcoholism. It can also be used as a life saving drug in case of paracetamol poisoning. It is chemically *N*-acetyl-*L*-cysteine1 (Fig. 1).

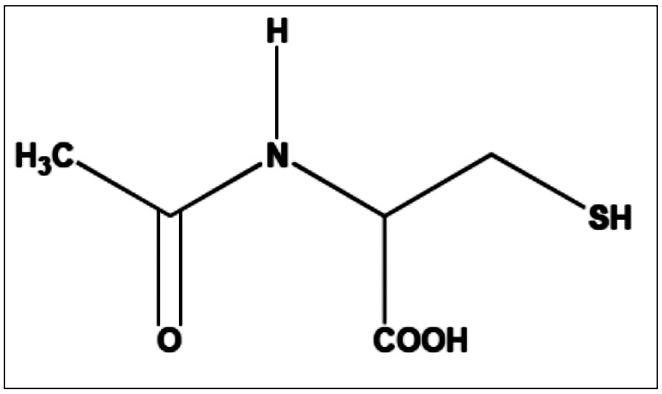

Fig. 1: Chemical structure of ACE

Ambroxol hydrochloride (AMB) is a secretolytic agent used in the treatment of the upper respiratory tract. It helps in mucus clearing and reducing cough. Its chemical name is trans-4-((2-amino-3, 5,-di bromobenzyl) amino) cyclohexanol hydrochloride[1] (Fig. 2).

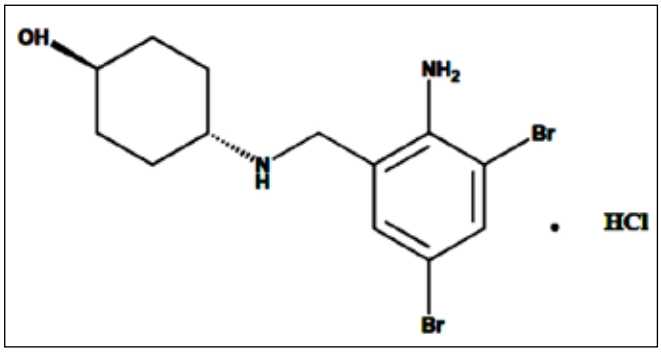

Fig. 2: Chemical structure of AMB

The combination of ACE and AMB is used effectively as an anti-oxidant and as an anti-inflammatory medicine.

The stability indicating method is defined as a validated quantitative analytical method that can detect the change with time in the chemical, physical or microbiological properties of the drug substance and the drug product, that are specific so that the content of active ingredient, degradation can be accurately measured without interference[2]. Stability testing gives information about degradation mechanism, possible degradation products, probable degradation pathways of the drug as well as interaction involving the drug and the excipients in the drug product[3].

[a] Department of Chemistry, Gudlavalleru Engineering College, Gudlavalleru - 521 356, Andhra Pradesh, India
* For Correspondence: Email- haritha.potluri@gmail.com

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Literature survey revealed an analytical method for estimation of AMB by HPLC and spectrophotometric method[4]. Very few analytical methods have been reported in combination of AMB with other drugs like spectrophotometric method[5], RP-HPLC method[6] and RP-UPLC method[7]. ACE in combination with other drugs in human urine is estimated by liquid chromatography method[8]. No analytical method was available from the literature for simultaneous determination of ACE and AMB. The scope of the present investigation was to develop a simple, accurate, precise, consistent, selective, sensitive, stability indicating, experimental design based robustness method for the simultaneous determination of ACE and AMB in a bulk drug and also in combined pharmaceutical tablet formulation by RP-HPLC technique.

MATERIALS AND METHODS

The analytical quality samples of ACE (99.68%) and AMB (99.65%) were received as gift samples from Unimarck Pharma (India) Ltd. Tablets of ACE and AMB were procured from the local stores. Milli-Q water, HPLC grade orthophosphoric acid, methanol and acetonitrile was purchased from SD Fine Chemicals Ltd., India.

Apparatus and chromatographic condition

The chromatographic separation was achieved on Waters HPLC incorporated with auto sampler and UV detector. Kromosil (250 mm x 4.6 mm, 5 µ) C18 column was used for the chromatographic separation. The mobile phase consisted of ortho phosphoric acid (0.1%**)** and acetonitrile in the ratio of 28:72 at a temperature of 30 °C. The mobile phase was prepared freshly, filtered, sonicated before use and delivered at a flow rate of 1.0 mL min^{-1} and the detector wavelength was set at 274 nm. The volume of injection was 10 µL. The diluent used was methanol and the final dilution was done using water: acetonitrile taken in the ratio of 50:50. Data was collected using Empower 2 software.

Preparation of standard ACE and AMB

Accurately weighed 20 mg of ACE and 15 mg of AMB working standards were transferred into 10 mL and 50 mL clean dry volumetric flasks separately and 15 mL of diluent was added. The solution was sonicated for 30 minutes and made up to the final volume with diluents. 1 mL each of the above stock solutions was pipetted out into a 10 mL volumetric flask and then made up to the final volume with diluents.

Sample solution preparation of ACE and AMB

Twenty tablets, of which the labeled claim was 200 mg of ACE and 30 mg of AMB per tablet, were taken and powdered very finely. Accurately weighed sample containing 200 mg of ACE and 30 mg of AMB was transferred into a 100 mL clean and dry volumetric flask and about 75 mL of diluent was added and sonicated to dissolve it completely and made up to the mark by using the diluents and labeled as sample stock solution. This was filtered by using the HPLC filters of 0.45 µ porosity and then 1 mL of filtered sample stock solution was transferred to 10 mL volumetric flask and made up to the mark with diluents.

A volume of 10 µL each of standard and sample solutions containing ACE and AMB, was injected into the chromatographic system and from the obtained peak areas, the % assay value was calculated.

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Chromatographic behavior using various columns like Hypersil-BDS-C18, Symmetry C18, Ymc-pack C18, Kromosil[8], Spherisorb C18, Phenomenex C18 have been tried with different compositions of the mobile phase. However less tailing and high theoretical plates are obtained with Kromosil (250 mm x 4.6 mm, 5 µ) C18 column at 274 nm of UV detection. A composition of 0.1% ortho phosphoric acid and acetonitrile in the ratio of 28:72 (V/V) was used as the mobile phase with a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 30°C. At the reported composition of mobile phase, peak shape was excellent; however altering the mobile phase composition resulted in unacceptable tailing factor and poor peak shape. Hence 0.1% ortho phosphoric acid and acetonitrile in the ratio of 28:72 (V/V) was optimized mobile phase.

RESULTS AND DISCUSSION

The analytical validation parameters for the proposed method are elucidated according to the guidelines of ICH9. The validation parameters achieved are summarized in Table I.

Linearity

The linearity for the proposed HPLC method was established at six concentration levels, ranging from 50-300 µg mL^{-1} for ACE and 7.5-45 µg mL^{-1} for AMB**.** The calibration curve was constructed by plotting response factor against respective concentration of ACE and AMB. From the plots constructed between peak area Vs respective concentration of the drugs, the linearity was

Table I: Analytical validation parameters (system suitability and linearity)

Parameter	ACE	AMB
Linearity	50-300µg mL^{-1}	7.5-45 µg mL^{-1}
Slope	10308	21813
Intercept	859.61	1576.3
r^2	0.9995	0.9997
LOD	0.04µg mL^{-1}	0.14µg mL^{-1}
LOQ	0.12µg mL^{-1}	0.44µg mL^{-1}
Theoretical Plates	2101324	664302
Tailing Factor	1.02	1.19
Retention Time (min)	2.401	5.800

established in the range of 50 - 300 µg mL^{-1} and 7.5 - 45 µg mL^{-1} for ACE and AMB, respectively. Linear regression analysis was used for the establishment of linearity and coefficient of correlation (r2) and it was found to be 0.999 for both the drugs.

Recovery

Three different samples of known concentrations in the given range for ACE and AMB were prepared and analyzed against standard solution. The recovery analysis study data of ACE and AMB was done and the obtained results are represented in Table II.

Sensitivity

The lowest concentration of the drug that gives response is termed as limit of detection (LOD). The lowest concentration analyzed with accuracy by the proposed RP-HPLC method is termed as limit of quantification (LOQ). The values for the limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.04 µg.mL^{-1} and 0.12 µg.mL^{-1} for ACE, and it was 0.14 µg.mL^{-1} and 0.44 µg.mL^{-1} for AMB, respectively. The LOD and LOQ showed the sensitivity of the method for ACE and AMB.

Table II: Recovery studies of ACE and AMB

Recovery data of ACE					
Concentration (at specified level)	**Peak area**	**Amount added in µg**	**Amount found in µg**	**% recovered**	**Mean % recovery**
50 %	3093226.7	100	99.74	99.71	100.54
100 %	4139322.7	200	201.09	100.54	
150 %	5201513.3	300	304.04	101.34	
Recovery data of AMB					
Concentration (at specified level)	**Peak area**	**Amount added in µg**	**Amount found in µg**	**% recovered**	**Mean % recovery**
50 %	984790.33	15	15.06	100.49	100.40
100 %	1313160.3	30	30.12	100.41	
150 %	1640273	45	45.11	100.27	

Table III: Intra-day and Inter-day precision analysis of ACE and AMB

Drug	Concentration (μgmL^{-1})	Intra-day precision		Inter-day precision	
		SD	% RSD	SD	% RSD
ACE	200	24100.5	1.1	6829.5	0.3
AMB	30	3545.2	0.5	2812.6	0.4

Table IV: Assay result of tablet dosage form

Drug	Label strength in mg	Amount found in mg	% Assay
ACE	200	202.06	101.03
AMB	30	29.90	99.69

System suitability test

The specificity of this method was determined by complete separation of ACE and AMB as shown in Fig. 3 with parameters like retention time, resolution and tailing factor. Here tailing factor for peaks of ACE and AMB was less than 2 % and resolution was satisfactory. The average retention time for six replicate samples of ACE and AMB was found to be 2.401 and 5.800 minutes, respectively. The % RSD for retention

Table V: Forced degradation studies of ACE and AMB

Stress Conditions	Degradation Time	Peak Area		Degradation %		Active drug % present after degradation	
		ACE	AMB	ACE	AMB	ACE	AMB
Control		2135224	673807				
Acid	30 min	2049886	654028	4.00	2.94	96.00	97.06
Alkali	30 min	2070520	655364	3.04	2.74	96.96	97.26
Oxidative	30 min	2029087	650344	4.98	3.49	95.02	96.51
Thermal	6 hours	2123108	670652	0.57	0.47	99.43	99.53
Photo	7 days	2119418	671580	0.75	0.34	99.25	99.66

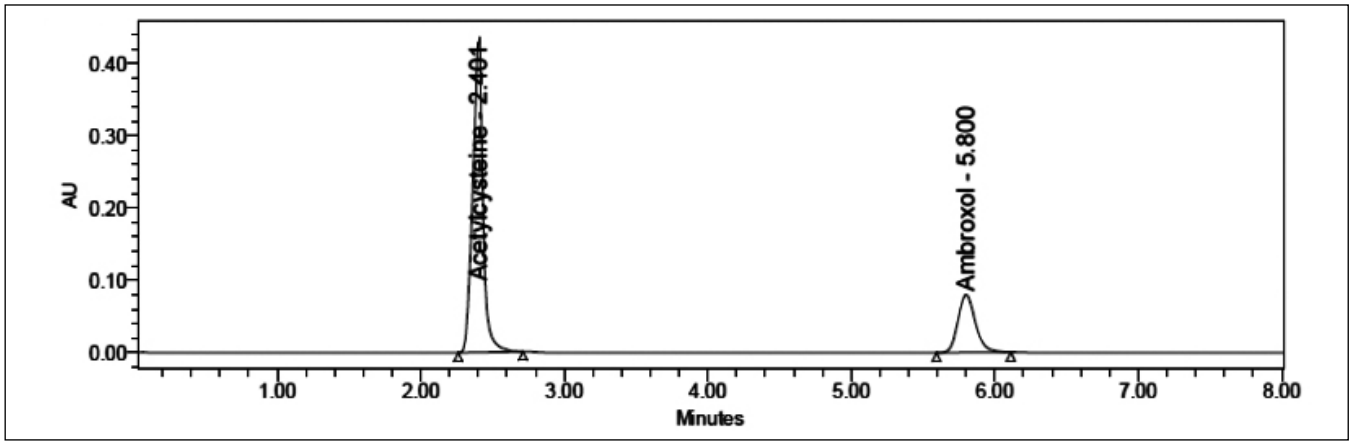
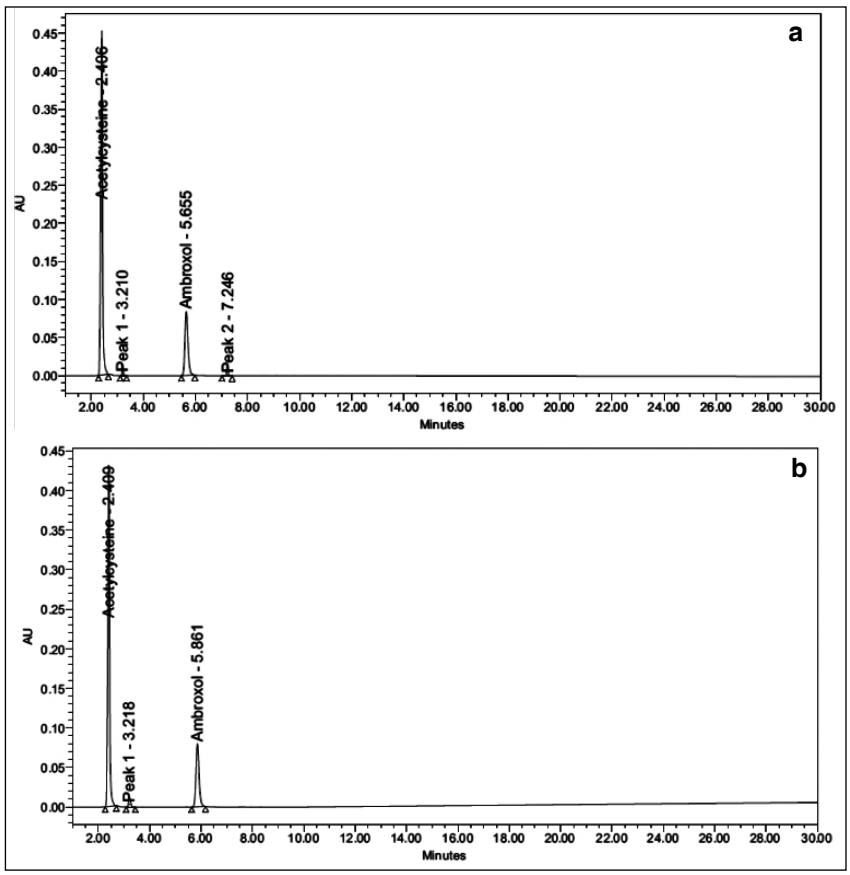


Fig. 3: Chromatogram of ACE and AMB



time was obtained as 0.06 and 0.08 for ACE and AMB, respectively. The peaks obtained for ACE and AMB were found to be sharp and have apparent baseline separation. Analysis was also performed for active ACE and AMB, placebo sample (all the ingredients except active ACE and AMB) both in stressed and unstressed conditions. After analysis, it was found that there is no interference of peak in the ACE and AMB region for the stressed, placebo & active sample. Hence it was emphasized that the developed method was specific for the analysis of this drug.

Precision

A study was carried out for intermediate precision with the same analyst on different days for six sample preparations of marketed formulations. The intra-day and inter-day precision analysis results are elucidated in Table III. The assay results of tablet dosage formulation by the proposed method are given in Table IV.

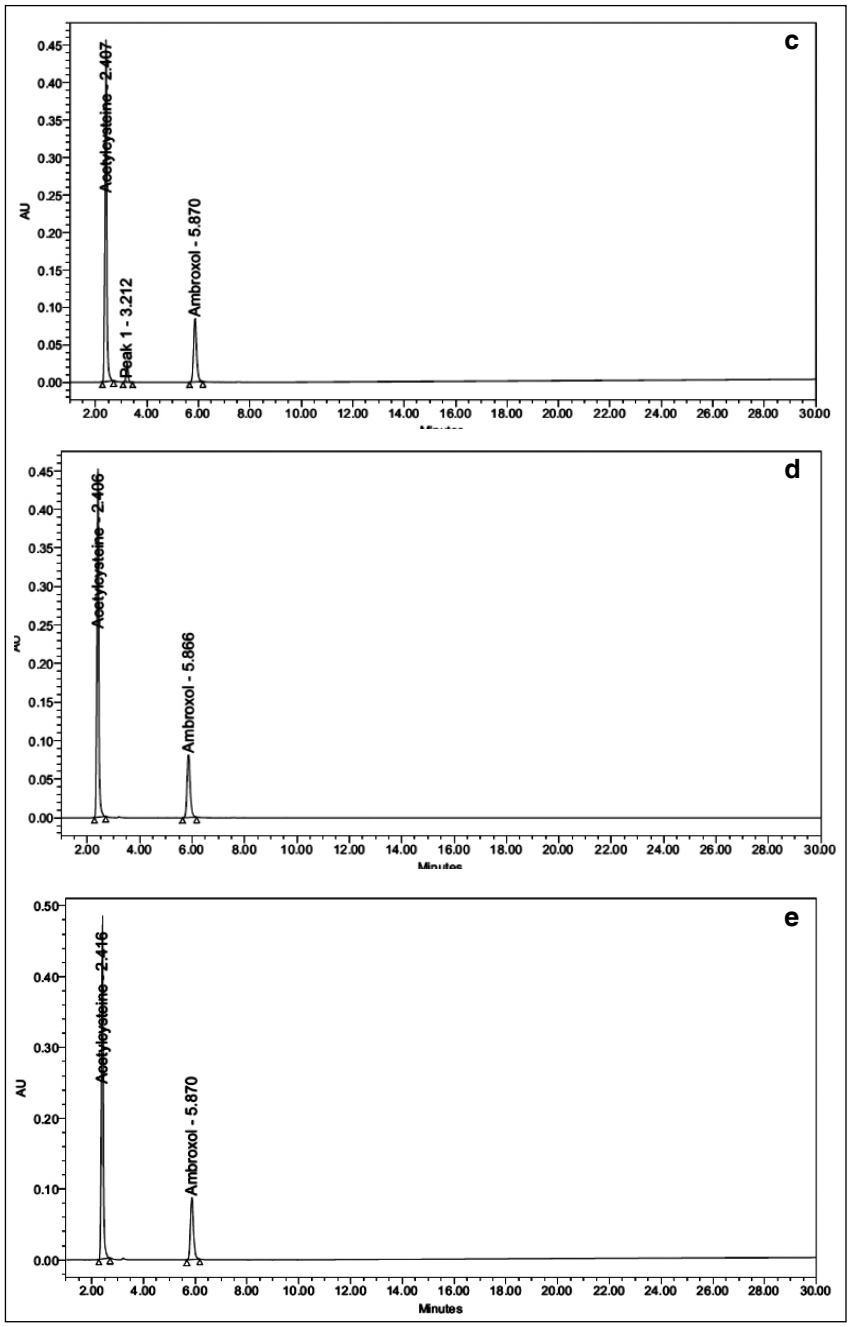


Fig. 4: a) Acid degradation studies chromatogram of ACE and AMB with degradation products (peak 1 and 2); b) Alkali degradation studies chromatogram of ACE and AMB with degradation products (peak 1); c) Oxidative degradation studies chromatogram of ACE and AMB with degradation product (peak 1); d) Thermal degradation studies chromatogram of ACE and AMB e) Photo degradation studies chromatogram of ACE and AMB.

Stability

For the purpose of demonstrating the stability of both standard and sample solutions during the period of analysis, both solutions were tested for a period of 24 hours at room temperature and the analysis showed that no considerable degradation has occurred.

Forced degradation studies

Forced degradation studies were conducted for indicating the stability of the developed method.

The standard solution equivalent to 200 mg of ACE and 30 mg of AMB was freshly prepared and was labeled as control sample. The study for stress degradation was done with 1 mL of standard solution under various conditions such as acid degradation studies (2N HCl at 60 °C for 30 min), alkali degradation studies (2N NaOH at 60 °C for 30 min), oxidative degradation studies (20 % H_2O_2 at 60 °C for 30 min). The standard drug solution was placed in an oven at 105 °C for about 6 hours for studying degradation due to heat and for studying photochemical degradation, the standard solution was kept in UV Chamber for 7 days. The solution was then diluted to obtain 200 µg mL^{-1} of ACE and 30 µg mL^{-1} of AMB solution. Then a 10µL of this solution was injected into the system and the chromatograms recorded to evaluate the stability of sample. Results of the degradation studies are presented in Table V. The chromatograms obtained for various degradations are represented in Fig. 4.

ROBUSTNESS

As per ICH guidelines[9], robustness study was performed to establish the potentiality of the developed method for slight changes in the method conditions like flow rate (1.0 ± 0.2 mL min-1), buffer composition in mobile phase (28 ± 2.8%) and column oven temperature (30 ± 5°C). No substantial effect was observed on retention times of analytes with these variations. The results are shown in Table VI.

Experimental Design Methodology

Design expert is used as software for evaluation of experimental design study (Stat-Ease Inc, Minneapolis, USA, version 10.0). Minimum measures are sufficient for experimental design than regular one-at-a-time procedure for providing same precision. Also, it gives the information of interfaces between the factors. In order to create the real-time difference of the desired factors on the measured

Table VI: Retention times of analytes in robustness conditions

Parameter	RT of ACE	RT of AMB
System suitability	2.401	5.800
Flow rate -1.2 mL.min^{-1}	2.283	5.588
Flow rate -0.8 mL.min^{-1}	2.573	6.272
Column temperature – 35 °C	2.406	5.643
Column temperature – 25 °C	2.408	5.652
Buffer composition in mobile phase (30.8 %)	2.407	6.336
Buffer composition in mobile phase (25.2 %)	2.410	5.642

responses, an approach of design of experiments was employed for robustness testing10,11. Due to high competence arising with limited number of runs, full factorial model was used for studying robustness. Three variables are taken at two levels, and a 23 full factorial design was employed for robustness testing. Retention times of both the analytes were measured as responses. Linear model ANOVA was employed to conclude the model coefficients and also to evaluate the robustness capability of the method.

The effects of the three factors in the retention times of ACE and AMB were represented in Fig. 5 (a) and 5 (b) for Pareto chart, respectively. Flow rate in mL.min^{-1} is represented as (A), column temperature is represented as

Table VII: The levels and range of the variables in the 23 full factorial design

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A:Flow mL. min^{-1}	B:Temperature ^{0}C	C:% Buffer in Mobile phase	RT of Acetylcysteine	RT of Ambroxol
1	1	0.8	25	25.2	2.734	6.213
2	4	1.2	25	25.2	2.289	5.149
3	3	0.8	35	25.2	2.611	6.121
4	7	1.2	35	25.2	2.278	5.302
5	6	0.8	25	30.8	2.654	6.875
6	10	1.2	25	30.8	2.333	5.871
7	5	0.8	35	30.8	2.599	6.79
8	8	1.2	35	30.8	2.247	5.797
9	2	1	30	28	2.401	5.8
10	9	1	30	28	2.392	5.832

Table VIII: 23 full factorial design- ANOVA data for response: retention time of ACE

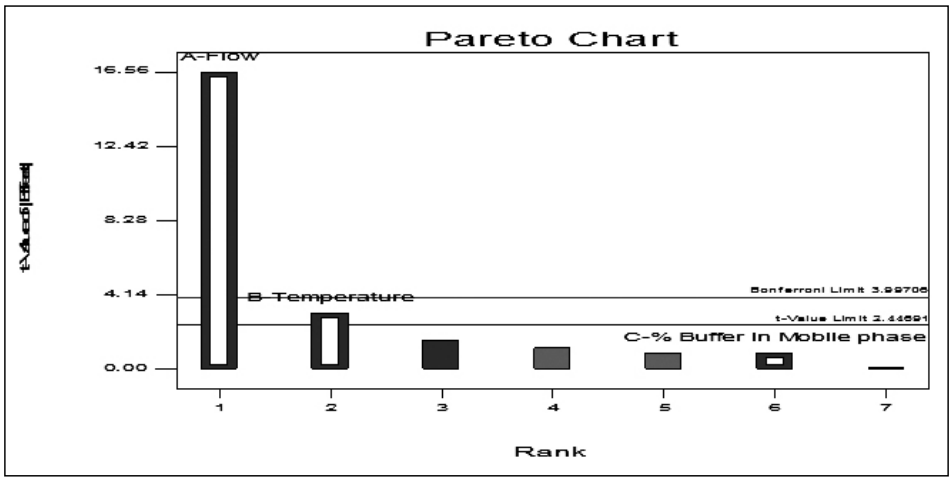
	Sum of		Mean	F	p-value	
Source	squares	df	square	value	Prob> F	
Model	0.27	3	0.091	42.03	0.0002	significant
A-Flow rate	0.26	1	0.26	121.38	< 0.0001	
B-Temperature	9.453E-003	*1*	9.453E-003	4.36	0.0818	
C-% Buffer	7.801E-004	*1*	7.801E-004	0.36	0.5706	
Residual	0.013	6	2.168E-003			
Lack of Fit	0.013	*5*	2.594E-003	64.04	0.0946	not significant
Pure Error	4.050E-005	*1*	4.050E-005			
Cor Total	0.29	9				

df: degree of freedom, Cor Total: corrected total sum of squares, Values of Prob> F less than 0.05 represents that the models are significant.

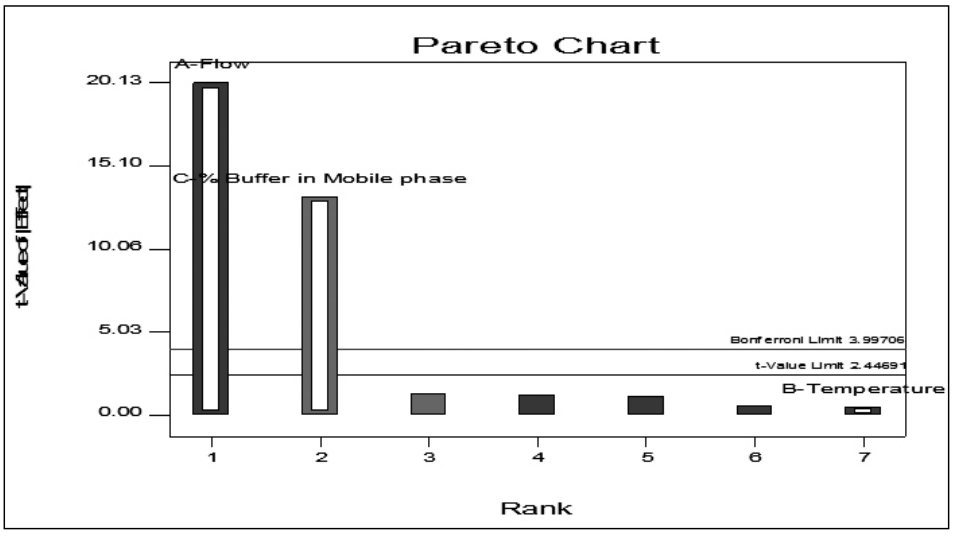
Table IX: 23 full factorial design-ANOVA data for response: retention time of AMB

	Sum of		Mean	F	p-value	
Source	squares	df	square	value	Prob> F	
Model	2.69	3	0.90	62.36	< 0.0001	significant
A-Flow rate	1.88	1	1.88	130.64	< 0.0001	
B-Temperature	1.200E-003	1	1.200E-003	0.083	0.7825	
C-% Buffer	0.81	1	0.81	56.34	0.0003	
Residual	0.086	6	0.014			
Lack of Fit	0.086	5	0.017	33.56	0.1303	not significant
Pure Error	5.120E-004	1	5.120E-004			
Cor Total	2.78	9				

df: degree of freedom, Cor Total: corrected total sum of squares, Values of Prob> F less than 0.05 represents that the models are significant.



(a)



(b)

Fig. 5: Pareto chart representation of factor (a) A >B >C for retention time of ACE (b) A >C>B for retention time of AMB

(B) and Buffer composition percentage in mobile phase is represented as (C).The factors and responses for the trials are shown in Table VII.

Standard and sample solutions are injected into chromatograph system. The retention times were studied as responses. The significance and contribution of the factors was estimated by statistical ANOVA, by ordering the F-ratio. Significance of the model was evaluated by the higher F-value that resembles like smaller "Prob>F" value. Table VIII and Table IX show the data of ANOVA analysis for retention times of ACE and AMB, respectively. The p-value and F-value of the model for ACE are 0.0002 and 42.03, respectively and that for AMB are 0.0001 and 62.36, respectively, demonstrating that the predicted model fits to the experimental data satisfactorily. ANOVA analysis data also indicate that, the temperature and % buffer in mobile phase does not have any effect on the retention time of ACE and the temperature does not have any effect on the retention time of AMB.

Thus, with a slight change in mobile phase flow, column temperature, percentage buffer in the mobile phase throughout analysis, the retention times of analyte will be within the defined run time and if the flow rate increases, then retention time of analyte decreases. Also, as the percentage of buffer composition increases, the retention time of AMB increases.

Design space

Design space concept was explained by the experimental design approach, the multi dimensional arrangement and interaction of input values and process factors that have explained to provide the maximum output of quality. Out of value for a design space is reflected by a change and usually initiates a post approval regulatory process. Design space is anticipated by the applicant and is dependent on regulatory assessment. Design space was recognized by applying the variables of full factorial design, defined as flow rate (A) 0.8-1.2 mL min^{-1}, temperature of column as (B) 25–35 °C and buffer composition in mobile phase (C) 25.2-30.8%.

CONCLUSION

This investigation presents a simple and validated RP-H stability indicating HPLC method for simultaneous estimation of ACE and AMB in the presence of degradation products. Experimental design based evaluation using a 23 full factorial design was applied to evaluate coefficient, ANOVA for the establishment of robustness nature of the method. The method developed was accurate, precise, sensitive, specific, and robust. The degradation products that are formed during the exposure of the drug to stress conditions gave peaks that are well separated from the peaks of the analytes, which establishes that the developed method was specific and stability indicating. The developed method can be used successfully for determination of marketed formulations containing ACE and AMB.

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