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Development and validation of a stability indicating RP-HPLC method for the simultaneous determination of related substances of albuterol sulfate and ipratropium bromide in nasal solution

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ABSTRACT

A simple, sensitive and specific RP-HPLC method was developed for the quantification of related impurities of albuterol sulfate (AS) and ipratropium bromide (IB) in liquid pharmaceutical dosage form. The chromatographic separation employs gradient elution using an inertsil C8-3, 250 mm × 4.6 mm, 5 μm columns. Mobile phase consisting of solvent A (solution containing 2.5 g of potassium dihydrogen phosphate and 2.87 g of heptane-1-sulfonic acid sodium salt per liter of water, adjusted to pH 4 with orthophosphoric acid) and solvent B (acetonitrile) delivered at a flow rate of 1.0 ml min^{-1}. The analytes were detected and quantified at 210 nm using photodiode array (PDA) detector. The method was validated as per ICH guidelines, demonstrating to be accurate and precise (repeatability and intermediate precision level) within the corresponding linear range of known impurities of AS and IB. The specificity of the method was investigated under different stress conditions including hydrolytic, oxidative, photolytic and thermal as recommended by ICH guidelines. Relevant degradation was found to take place under hydrolytic and oxidative conditions. Robustness against small modification in pH, column oven temperature, flow rate and percentage of the mobile phase composition was ascertained. Lower limit of quantification and detection were also determined. The peak purity indices (purity angle < purity threshold) obtained with the aid of PDA detection and satisfactory resolution between related impurities established the specificity of the determination. All these results provide the stability indicating capability of the method.

1. Introduction

Racemic (R, S)—albuterol is 1:1 mixture of R-enantiomer which has bronchodilatory and anti-inflammatory effects, and S-enantiomer which is associated with increased airway hyperreactivity and pro-inflammatory effects [1]. Ipratropium bromide (IB) (also known as Sch 1000) is a new atropine-like bronchodilator drug, whose mechanism of action is via an anticholinergic pathway and may decrease cyclic guanosine monophosphate [2]. Ipratropium nasal spray is safe, effective way to treat chronic rhinorrhea in laryngectomized patients, improving their quality of life [3]. The potential of anticholinergics to provide bronchodilatory benefits over short-acting β_2-agonists (SABA) alone in patients with moderate-to-severe persistent asthma has not been well defined. AS + IB resulted in significantly greater improvement in forced expiratory volume in 1 s (FEV_1) and longer duration of response compared to AS alone in patients with moderate-to-severe persistent asthma [4].

The literature survey reveals that, AS is reported in USP [5] while it is reported as salbutamol in EP [6]. Few HPLC methods are reported for the determination of salbutamol or albuterol [7], related substances [8–11] and enantiomeric separation [12]. The other methods available in the literature are based on CE [13], LC–MS [14] GC–MS and [15]. Articles on the determination of IB and related substances by HPLC [16,17] and spectrophotometric methods [18] are very few.

However the exhaustive literature survey revealed that none of the most recognized pharmacopoeias or any journals includes these drugs in combination for the simultaneous determination of related substances of AS and IB and the information regarding the stability of the drugs is not available. So it is felt essential to develop a liquid chromatographic procedure which will serve a reliable, accurate, sensitive and stability indicating HPLC method for the simultaneous determination of related substances of AS and IB in AS + IB nasal solution.

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Table 1
Chemical names of all related impurities of AS and IB.

Compounds	Chemical name
Albuterol sulfate and its related impurities	
Albuterol sulfate	α^1-[(Tert-butylamino) methyl]-4-hydroxy-m-xylene-α, α'-diol sulfate
Impurity-B	(1RS)-2-[(1,1-dimethylethyl) amino]-1-(4-hydroxyphenyl) ethanol
Impurity-C	(1RS)-2-[(1,1-dimethylethyl) amino]-1-(4-hydroxy-3-methylphenyl) ethanol
Impurity-D	5-[(1RS)-2-[(1,1-diethylethyl) amino]-1-hydroxyphenyl]-2-hydroxy benzaldehyde
Impurity-E	(1RS)-2-[benzyl (1,1-dimethylethyl) amino]-1-[4-hydroxy-3-(hydroxyl methyl) phenyl] ethanol
Impurity-F	1,1′-[Oxybis [methylene (4-hydroxy-1,3-phenylene)]] bis [2-[(1,1-dimethyl ethyl) amino] ethanol]
Impurity-G	2-[Benzyl (1,1-dimethylethyl) amino]1-1[4-hydroxy-3-(hydroxymethyl) phenyl] ethanone
Impurity-H	4-[2-[(1,1-Dimethylethyl) amino] ethyl]-2-methylphenol
Impurity-I	(1RS)-2-[(1,1-dimethylethyl) amino] 1-1[3-(hydroxymethyl)-4-benzyloxy phynyl] ethanol
Impurity-J	2-[(1,1-Dimethylethyl) amino]-1-[4-hydroxy-3-(hydroxymethyl)ethanone (salbutamone)]
Ipratropium bromide and its related impurities	
Ipratropium Br	[(1S, 5R)-8-methyl-8-propan-2-yl-8-azoniabicyclo [3.2.1] octan-3-yl] 3-hydroxy-2-phenylpropanoate bromide
Impurity-B	8s-Ipratropium
Impurity-C	DL-tropic acid
Impurity-D	2-Phenyl propenoic acid (atropic acid)
Impurity-E	Isopropyl notropine
Impurity-F	Apo-ipratropium

Regulatory agencies recommend the use of stability indicating methods (SIMs) [19] for the analysis of stability samples [20]. This requires stress studies in order to generate the potential related impurities under stressed conditions, method development and validation [21]. With the evident of the International Conference on Harmonization (ICH) guidelines [22], requirements for the establishment of SIMs have become more clearly mandated.

Environmental conditions including light, heat and the susceptibility of the drug product towards hydrolysis or oxidation can play an important role in the production of potential impurities. Stress testing can help identifying degradation products and provide important information about intrinsic stability of the drug product.

Therefore, herein we report the results of stability study of AS and IB with the aim of determining the extent of the influence of different stress conditions on the stability of drug product.

2. Experimental

2.1. Reagents and materials

AS and IB active pharmaceutical ingredient (API) and test sample solution (each 3 ml of nasal solution contains 3 mg of AS and 0.5 mg of IB) were kindly supplied by Research and Development Centre, Wockhardt Limited, Aurangabad, India. The related substances of AS and IB (Fig. 1) were procured from Neuland Laboratories Limited, Hyderabad, India. The chemical names for all components are listed in Table 1. Potassium dihydrogen orthophosphate, heptane-1-sulfonic acid sodium salt, orthophosphoric acid and HPLC grade acetonitrile were obtained from Merck Limited, Mumbai, India. High purity deionised water was obtained from Millipore, Milli-Q (Bedford, MA, USA) purification system.

Table 2
Mobile phase program for gradient elution.

Time (min)	Flow (ml min^{-1})	Solvent A (%)	Solvent B (%)
0	1.0	85	15
40	1.0	80	20
50	1.0	75	25
60	1.0	75	25
65	1.0	65	35
85	1.0	50	50
87	1.0	85	15
95	1.0	85	15

2.2. Instrumentation

HPLC system (Waters Milford, USA) equipped with inbuilt autosampler and quaternary gradient pump with an on-line degasser was used. The column compartment having temperature control and photodiode array (PDA) detector was employed throughout the analysis. Chromatographic data was acquired using empower software.

2.3. Chromatographic conditions

Inertsil C8-3 (250 mm × 4.6 mm), 5 μm (GL sciences Inc., USA) column was used as stationary phase maintained at 30 °C. The mobile phase involved a variable composition of solvent A (2.5 g potassium dihydrogen phosphate and 2.87 g heptane-1-sulfonic acid sodium salt dissolved in 1000 ml of water, adjusted to pH 4 with orthophosphoric acid) and solvent B (acetonitrile). The mobile phase was pumped through the column with at a flow rate of 1 ml min^{-1} (Table 2).

The optimum wavelength selected was 220 nm which represents the wavelength of maximum response for all impurities in order to permit simultaneous determination of related impurities of AS and IB in AS + IB nasal solution. The stressed samples were analyzed using a PDA detector covering the range of 200–400 nm.

2.4. Solution preparation

2.4.1. System suitability solution

Solution containing a mixture of 0.6 mg ml^{-1} of AS, 0.1 mg ml^{-1} of IB and 0.003 mg ml^{-1} of albuterol impurity-J was prepared in water.

2.4.2. Standard solution

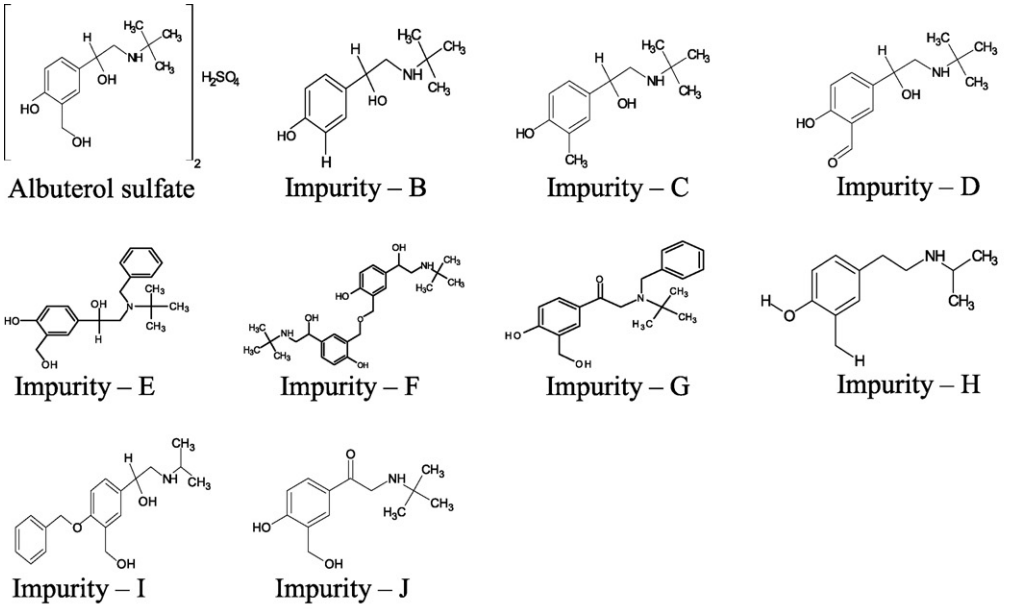
Solution containing a mixture of 6 μg ml^{-1} of AS and 1 μg ml^{-1} of IB working standard was prepared in water.

2.4.3. Sample solution

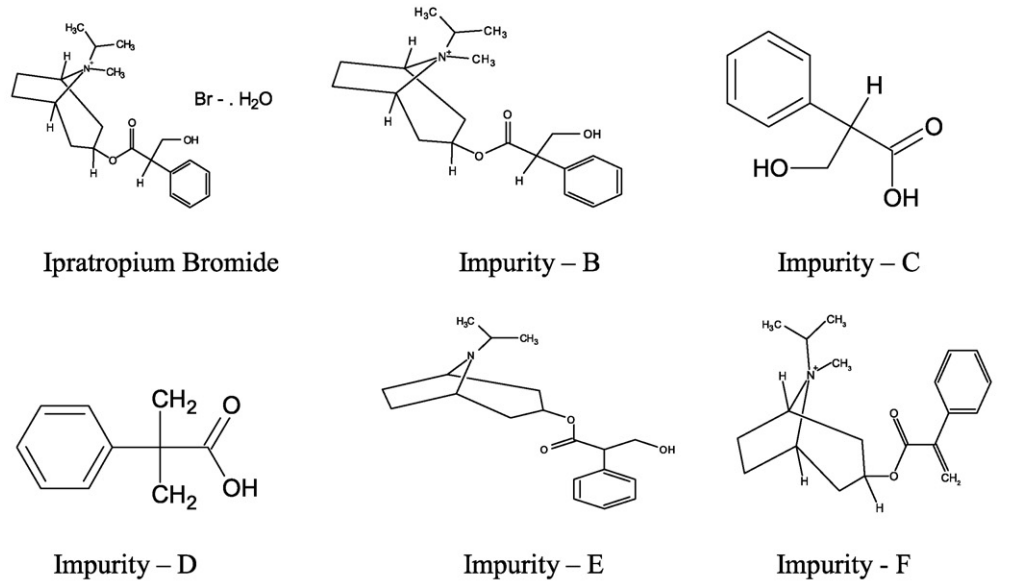
15 ml of AS + IB nasal solution (equivalent to 15 mg of AB and 2.5 mg of IB) was transferred to 25 ml volumetric flask and diluted to volume with water.

2.4.4. Forced degradation sample solution for specificity study

Multiple stressed samples were prepared as indicated below. They were chromatographed along with a non-stressed sample.



(a) Chemical structure of Albuterol sulfate and its related substances



(b) Chemical structure of Ipratropium bromide and its related substances

Fig. 1. Chemical structure of albuterol sulfate, ipratropium bromide and their respective impurities.

2.4.4.1. Hydrolytic conditions: acid-, base-induced degradation. Solution containing 0.6 mg ml^{-1} of AS and 0.1 mg ml^{-1} of IB was treated with 5N HCl and 0.1N NaOH respectively. These were subjected to the condition mentioned in Table 3. The solutions were neutralized as needed (5N NaOH or 0.1N HCl).

Table 3
Hydrolytic, oxidizing thermal and photolytic stress conditions.

Condition	Conditions	Time	Temperature (°C)	%Degradation
Acidic	5.0N HCl	30 min	80 °C	28.96
Basic	0.1N NaOH	10 min	60 °C	24.73
Oxidation	3% w/v H_2O_2	3 h	80 °C	2.30
Thermal	–	18 h	80 °C	Stable
Photolytic	250 Wh/m^2	22 h	–	Stable

2.4.4.2. Oxidative condition: hydrogen peroxide-induced degradation. Solution containing 0.6 mg ml^{-1} of AS and 0.1 mg ml^{-1} of IB was treated with 30% w/v H_2O_2 in dark under the condition shown in Table 3.

2.4.4.3. Thermal degradation study. 15 ml of AS + IB nasal solution (equivalent to 15 mg of AB and 2.5 mg of IB) was transferred in 25 ml volumetric flask and subjected to the oven under the conditions indicated in Table 3.

2.4.4.4. Photolytic degradation study. As per guidelines for photostability testing of new drug substances and products, samples should be exposed to light providing an overall illumination of not less than 1.2 million lx h and an integrated near ultraviolet energy of not less than 200 Wh/m^2 to allow direct comparisons to be made

between the drug substance and drug product [23]. AS + IB nasal solution (equivalent to 15 mg of AB and 2.5 mg of IB) was placed in a quartz vessel and exposed to forced irradiation (at 15 cm from the source) in a sun test chamber fitted with xenon lamp. Suntest meets the ID65 spectral criterion with an optical filter system consisting of a coated quartz glass dish, a window glass filter and an ID-65 filter used in combination. The suntest's filtered xenon light source is a full spectrum light source containing both visible and UV outputs with a UV cut-on of approximately 320 nm and a spectral distribution corresponding to ID65 per ISO 10977. The sample was exposed with 250 W/m^2 in suntest chamber for about 22 h which meets the total espouser of 1.2 million lx h (between 320 nm and 800 nm).

Similarly the common placebo (mixture of all excepients without both drugs), placebo with only AS and placebo with only IB were treated in same way as sample treated above in each conditions separately. All these stressed samples and placebos periodically analyzed by HPLC for the appearance of additional impurities and the impurities were identified on the basis of respective placebos and are calculated against the respective main drug component.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The possible impurities of AS and IB are very similar to respective drug substances. It is clear from the molecular structures (Fig. 1) that all the related compounds of AS are basic in nature and of IB are acidic in nature. To obtain a good resolution among the impurities and main drug substances different stationary phases were tested considering;

a. The feature of stationary phase (RP-C_8 and RP-C_{18}).
b. The particle size of the column (3 μm and 5 μm).

Considering that AS, IB and their related compounds are a mixture of acidic and basic in nature, we tested following mobile phases with gradient elution,

1. $KH_2PO_4{\cdot}H_2O$ (2.5 g L^{-1}) and heptane sulfonic acid sodium salt (2.87 g L^{-1}) as a buffer (pH 2.5, 3, 3.65, 4, 5) in combination with acetonitrile. The buffer pH 3.65 in combination with acetonitrile was used in the European pharmacopoeia 6.0 [6] to analyze related substances of salbutamol.
2. $KH_2PO_4{\cdot}H_2O$ (2.5 g L^{-1}) and heptane sulfonic acid sodium salt (2.87 g L^{-1}) as a buffer (pH 2.5) in combination with methanol.
3. $KH_2PO_4{\cdot}H_2O$ (2.5 g L^{-1}) and octane sulfonic acid sodium salt (3 g L^{-1}) as a buffer (pH 4) in combination with acetonitrile.
4. $(NH_4)H_2PO_4$ (1.15 g L^{-1}) and heptane sulfonic acid sodium salt (2.87 g L^{-1}) as a buffer (pH 3.8, 4, 4.2) in combination with acetonitrile.

3.1.1. Selection of stationary phase

It is clear from the molecular structure (Fig. 1) that all compounds do not possess a functional group which can readily ionize indicating non-polar in nature. But the presence of hydroxyl and amine groups shows some polar nature. Hence we started the development activity with C_{18} stationary phase of various manufacturers using different mobile phases. The poor resolution between albuterol and albuterol impurity-J and broad peak shape for albuterol implies that C_{18} stationary phase is not suitable for this application. Hence C_8 stationary phase was chosen to improve resolution among the peaks and peak shape for albuterol. The peak shape for albuterol and resolution among all components improved with inertsil C_8, 250 mm × 4.6 mm, 5 μm columns. But the stationary phase is not only the parameter which can give better separation among all impurities. Mobile phase, pH and organic modifies also plays very important role which leads to the best separation.

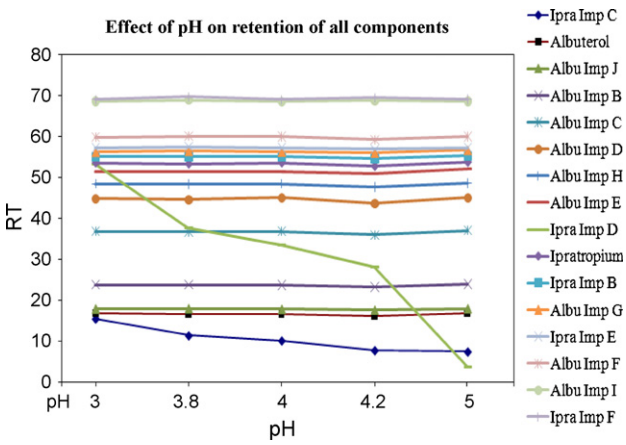


Fig. 2. Plot showing the influence of pH on retention of all components.

3.1.2. Influence of mobile phase buffer salt and surfactants

The resolution among the related impurities, AS and IB was found poor using mobile phase with octane-1-sulfonic acid sodium salt. Mobile phase containing heptane-1-sulfonic acid sodium salt with ammonium phosphate instead of octane-1-sulfonic acid sodium salt gives the better resolution. However one unknown impurity is merging with ipratropium impurity-D. The ammonium phosphate is now replaced with potassium phosphate buffer keeping heptane-1-sulfonic acid sodium salt as such, gives the better separation among the impurities.

3.1.3. Influence of organic modifier

Initially the methanol was used as an organic modifier which gives the poor baseline with baseline drift. Hence the response for the related compounds was reduced. The retention for all impurities was increased leading to poor resolution among the peaks. To improve the resolution among the peaks and response, acetonitrile was tried as an organic modifier. The base line was found good and response for all components was improved. The peak shape for all components was also improved and hence acetonitrile was selected as organic modifier.

3.1.4. Influence of pH of mobile phase buffer

The molecular structure of all components (Fig. 1) implies that all compounds do not possess any functional groups which can readily ionize. But ipratropium impurities C and D contains carboxylic acid functional group which can ionize and reflects the impact on separation with pH variation. The retention time and separation of all the components remains unchanged except ipratropium impurities C and D with change in mobile phase pH. These two impurities show the dramatic change in retention time with pH variation affecting the resolution among the impurities. We tried different mobile phase buffers pH ranging from 3 to 5 and monitored the movement of these two impurities and graphically represented it in Fig. 2. The best separation was achieved with pH 4.

After an extensive study, the method has been finalized on inertsil C_8, 250 mm × 4.6 mm, 5 μm using variable composition of solvent A: KH_2PO_4 (2.5 g L^{-1}), heptane sulfonic acid sodium salt (2.87 g L^{-1}) pH 4 with orthophosphoric acid and solvent B: acetonitrile as mobile phase (Table 1). The mobile phase pumped through the column at a flow rate of 1.0 ml min^{-1} and column compartment

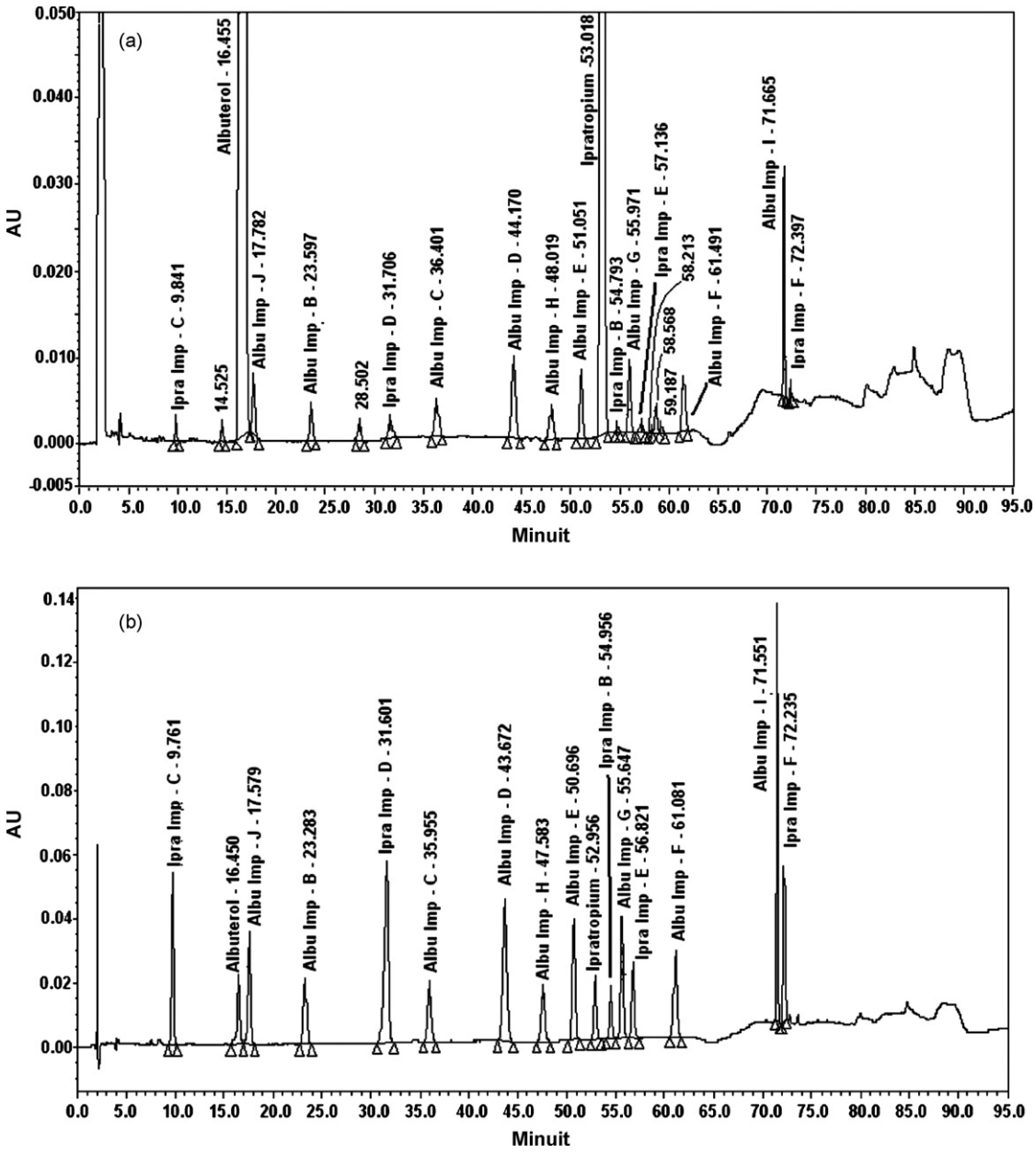


Fig. 3. Typical HPLC chromatogram of (a) spike sample and (b) mixture of all components.

temperature kept at 30 °C. The detector response for all the components found maximum at 210 nm; hence the typical chromatogram was recorded at this wavelength. The typical HPLC chromatograms (Fig. 3) represent the satisfactory separation of all components among each other.

3.2. Method validation

The optimized RP-HPLC method validated according to ICH guidelines [24], with respect to specificity, accuracy, precision (repeatability and intermediate precision), linearity, range and robustness. System suitability features were also assessed.

3.2.1. System suitability test

The system suitability test performed according to USP 30 [25] and BP 2007 [26] indications. The observed RSD values at 1% level of analyte concentration were well within the usually accepted values (≤2%). Theoretical plates, USP tailing factor (T_f) and USP resolution (R_s) between albuterol and albuterol impurity-J are also determined. The results obtained are all within acceptable limits.

3.2.2. Specificity

The peak purity indices for the analytes in stressed solutions were determined with PDA detector [27–29] under optimized chromatographic conditions found to be better (purity angle < purity threshold) indicating that no additional peaks were co-eluting with the analytes and evidencing the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference. Baseline resolution was achieved for all investigated compounds. The FDA guidelines indicated that well separated peaks, with resolution, $R_s > 2$ between the peak of interest and the closest eluting peak, are reliable for the quantification [30]. All the peaks meet this specification, visibly confirmed in Figs. 4–6. From Figs. 4–6 it was observed that in acid and base treatment the ipratropium impurity-C was formed as a major degradation impurity

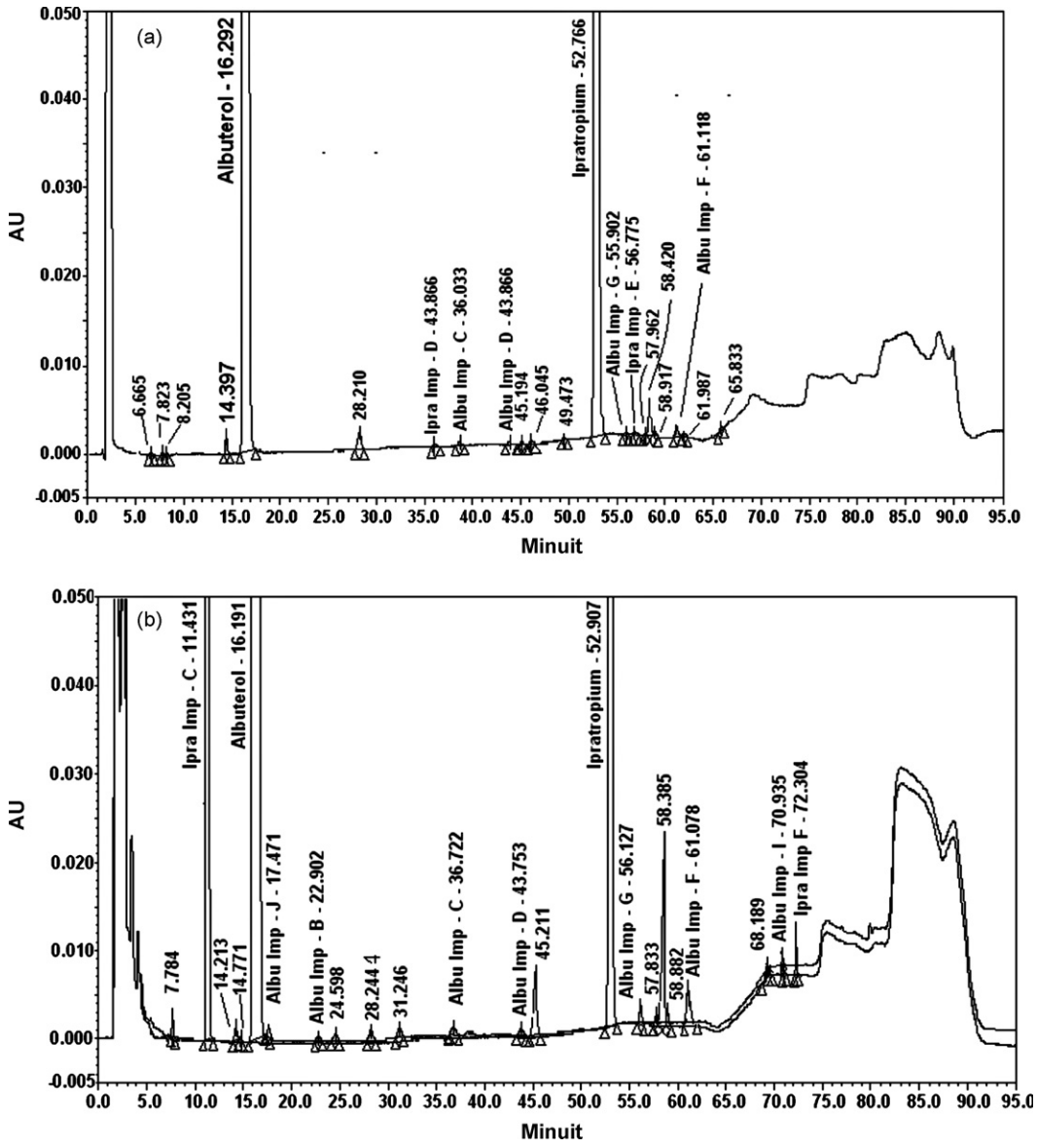


Fig. 4. Typical overlaid HPLC chromatograms of unstressed and stressed samples with placebo (a) untreated sample and (b) acid treated sample.

and few unknown impurities indicating that the drug product is very sensitive to acid and base.

3.2.2.1. Degradation kinetics under acidic and basic conditions. Sample solutions were treated with HCl of different normalities (1N, 2N and 5N) and 0.1N NaOH of different volumes (0.5 ml, 1 ml and 5 ml). The chromatograms obtained revealed that the peak area for AS remains unaffected while IB was reduced and ipratropium impurity-C was increased with increase in normality of HCl and volume of 0.1N NaOH respectively. The plots of respective components area against normality of HCl and volume of 0.1N NaOH indicated an apparent first-order degradation behavior (Fig. 7). In peroxide, thermal and photolytic conditions, the solution was found stable.

The stability of drugs in analytical solution was checked by preparing sample solution as per method and injected at regular time intervals in the proposed method at room temperature. On verifying the formation of additional peaks it was found that found that no additional peaks were formed till 26 h indicating that the sample solution is stable for about 26 h at room temperature.

3.2.3. Linearity and range

The nominal concentration of test solutions for AS and IB were 0.6 mg ml^{-1} and 0.1 mg ml^{-1}, respectively. Taking into account that typical impurity tolerance levels currently ranges between 0.1% and 0.3% for AS and IB and response function was determined by preparing standard solution of each component at different concentration levels ranging from lower limit of quantification to 120% of impurity tolerance level and that identification of impurities below lower level of quantification were not considered to be necessary unless the potential impurities are expected to be unusually potent to toxic.

The plots of area under the curve (AUC) of the peak responses of the analytes against their corresponding concentrations, they fitted straight lines responding to equations. The *y*-intercepts were close to zero with their confidence intervals containing the origin. The correlation coefficients (r) exceeds 0.98, the acceptance threshold suggested for linearity of procedures for the determination of impurity content in bulk drug [31]. Furthermore the plot of residuals exhibited random patterns with the residuals passing the normal distribution test ($p < 0.05$), all of which evidenced that the

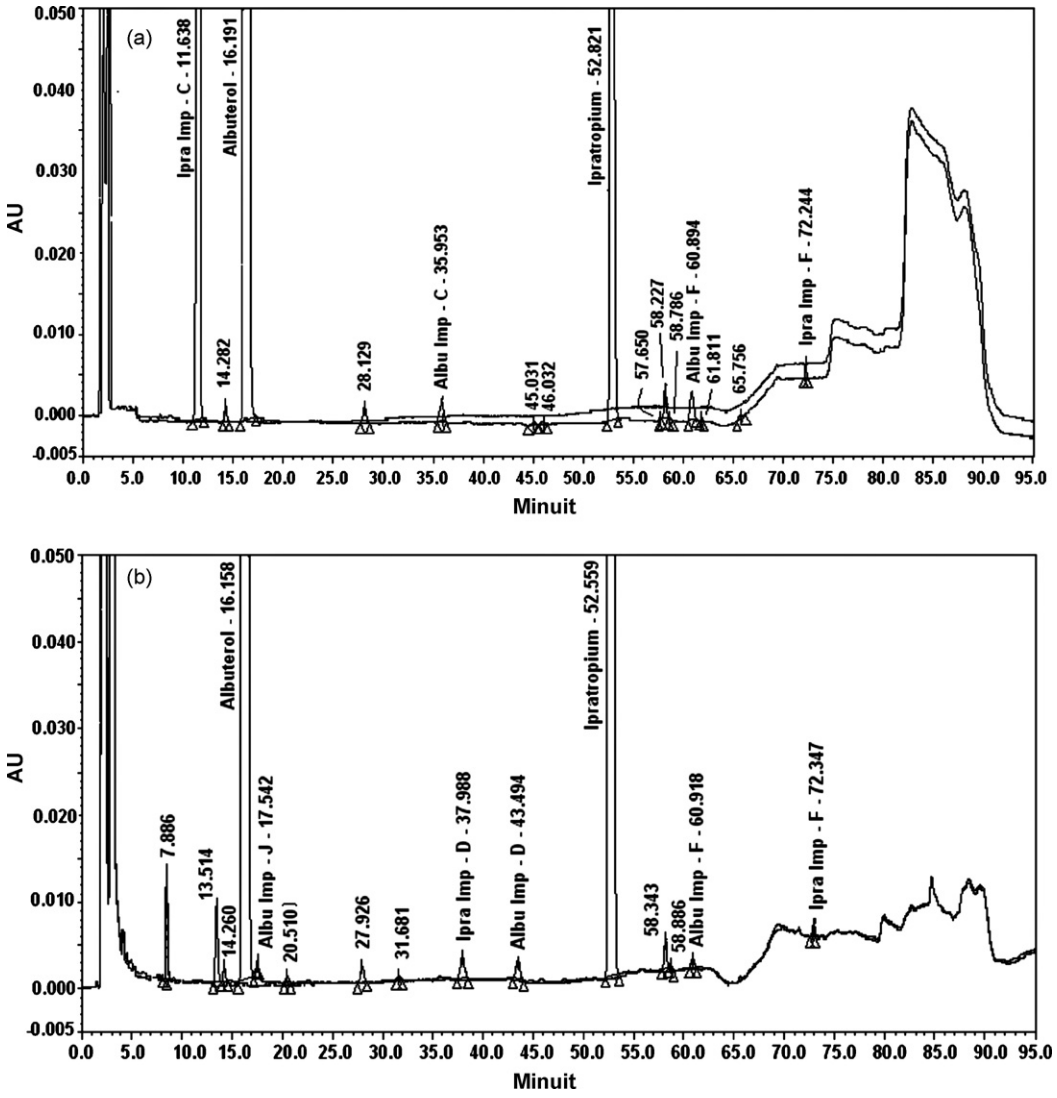


Fig. 5. Typical overlaid HPLC chromatograms of stressed samples with placebo (a) alkali treated sample and (b) peroxide treated sample.

Table 4
Regression statistics.

Comp.	Conc μg ml^{-1}	Multiple *R*	Regression equation	*t*-Stat	*P*-value	Lower	Upper
						95% confidence interval	
[A] Albuterol sulfate and its impurities							
AS	0.037–7.47	0.99999	$y = 132431.42x - 285.78$	903.20	6.17 E-37	132066.35	132691.15
Impurity-J	0.025–0.74	0.99993	$y = 218780.48x + 363.50$	288.53	1.09 E-22	217111.54	220449.42
Impurity-B	0.048–0.72	0.99991	$y = 165891.58x + 622.86$	220.53	4.12 E-18	164189.89	167593.27
Impurity-C	0.060–0.73	0.99988	$y = 183413.93x + 1281.78$	182.13	9.24 E-16	181101.54	185746.34
Impurity-D	0.036–0.73	0.99991	$y = 368056.95x + 755.89$	238.04	4.21 E-20	364611.86	371502.04
Impurity-H	0.036–0.72	0.99994	$y = 148357.23x - 171.90$	279.64	8.41 E-21	147175.13	149541.34
Impurity-E	0.036–0.73	0.99993	$y = 198163.66x - 0.65$	258.15	1.87 E-20	196453.27	199874.05
Impurity-G	0.12–0.72	0.99940	$y = 200243.13x - 7768.80$	76.76	1.68 E-11	194074.59	206411.67
Impurity-F	0.12–0.71	0.99869	$y = 170829.65x - 443.06$	51.55	2.71 E-10	162993.37	178665.92
Impurity-I	0.048–0.72	0.99994	$y = 271236.93x - 12.00$	282.89	4.39 E -19	269067.94	273404.92
[B] Ipratropium bromide and its impurities							
IB	0.010–1.24	0.99999	$y = 93416.19x - 46.08$	841.13	3.58 E-32	93176.26	93656.12
Impurity-C	0.018–0.37	0.99990	$y = 263541.50x + 938.75$	185.40	5.12 E-19	260374.30	266708.70
Impurity-D	0.030–0.36	0.99988	$y = 444022.39x + 0.94$	182.68	9.02 E-16	438417.41	449627.36
Impurity-B	0.060–0.36	0.99979	$y = 101231.78x - 663.02$	130.15	4.17 E-13	99412.53	103071.02
Impurity-E	0.061–0.37	0.99969	$y = 121038.52x + 155.91$	106.59	1.69 E-12	118353.27	123723.77
Impurity-F	0.024–0.36	0.99980	$y = 106843.33x - 800.11$	150.44	1.26 E-16	105236.73	108449.93

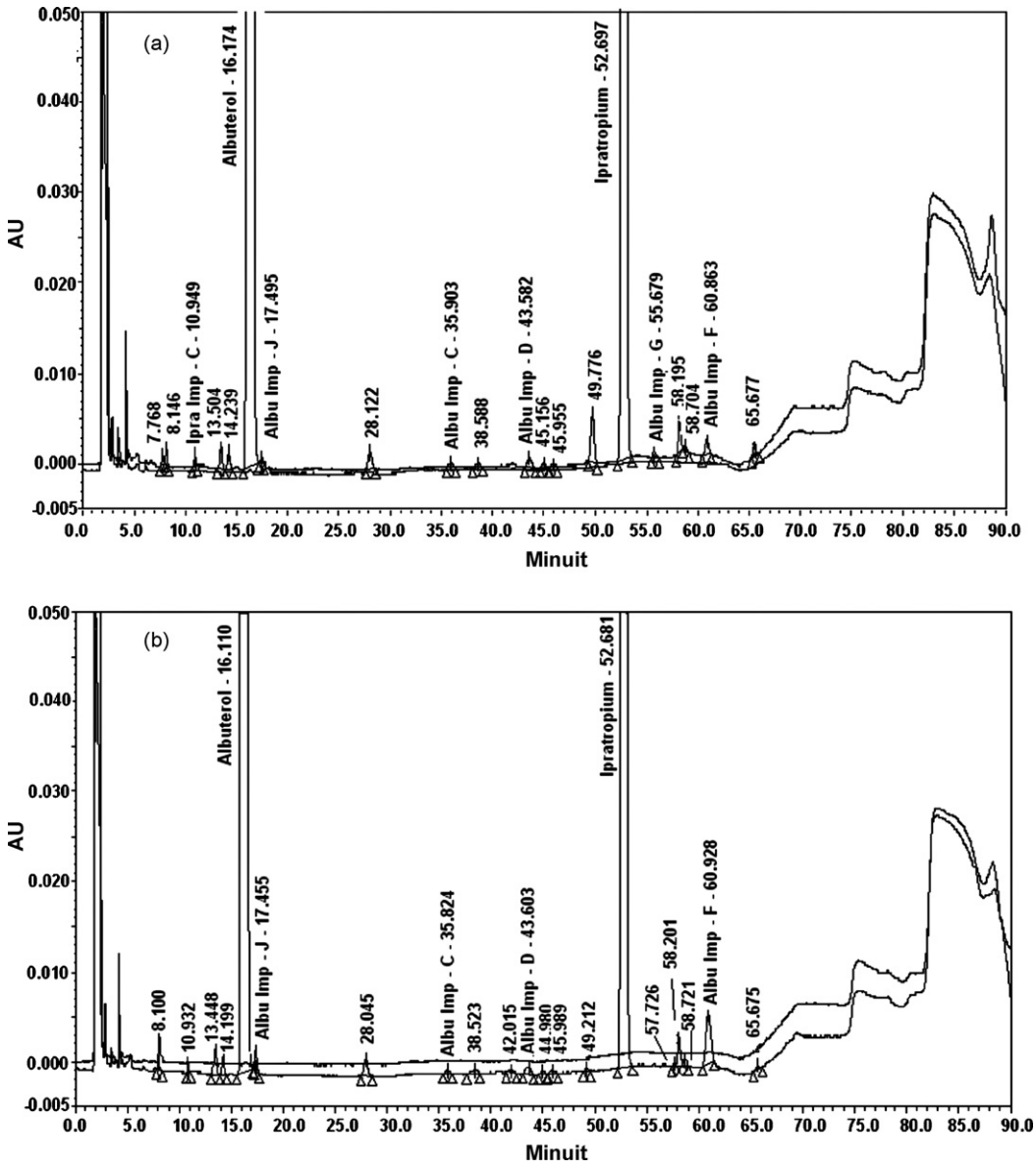


Fig. 6. Typical overlaid HPLC chromatograms of stressed samples with placebo (a) thermal treated sample and (b) sample exposed to light.

method is linear in the tested range. The regression statistics are shown in Table 4.

3.2.3.1. Determination of limit of quantification and detection (LOQ and LOD). The linearity performed above, used for the determination of limit of quantification and detection. Residual standard deviation (σ) method was applied and the LOQ and LOD values were predicted using following formulas (a) and (b) and established the precision at these predicted levels. The results are tabulated in Table 5.

$$\text{LOQ} = \frac{10\sigma}{S} \quad \text{(a)}$$

$$\text{LOQ} = \frac{3.3\sigma}{S} \quad \text{(b)}$$

where σ = residual standard deviation of response and S = slope of the calibration curve.

3.2.3.2. Determination of relative response factor (RRF) with linear calibration curve. The calibration curves for all components were constructed using the peak areas and analyte concentrations in the range mentioned in Table 4 by linear regression analysis. The linear regression equation containing slope for all components were summarized in Table 4. The RRF was determined as the ratio of slope of the regression line for each impurity to that of corresponding main drug components and is listed in Table 5.

3.2.4. Accuracy

Accuracy was evaluated by the simultaneous determination of analytes in solution prepared by standard addition method. The experiment was carried out by adding known amount of each related impurities corresponding to three concentration levels of 40%, 100% and 150% of the specification level in sample solution. The samples were prepared in triplicate at each level. The quantification of added analyte (%weight/weight) was carried out by using an external standard of corresponding main drug prepared at the analytical concentration. Relative response factors of all related impurities were used to calculate the weight percentage of related impurities in drug product. (Note: in routine analysis, the RRF of the related impurities that were either not tested in the method

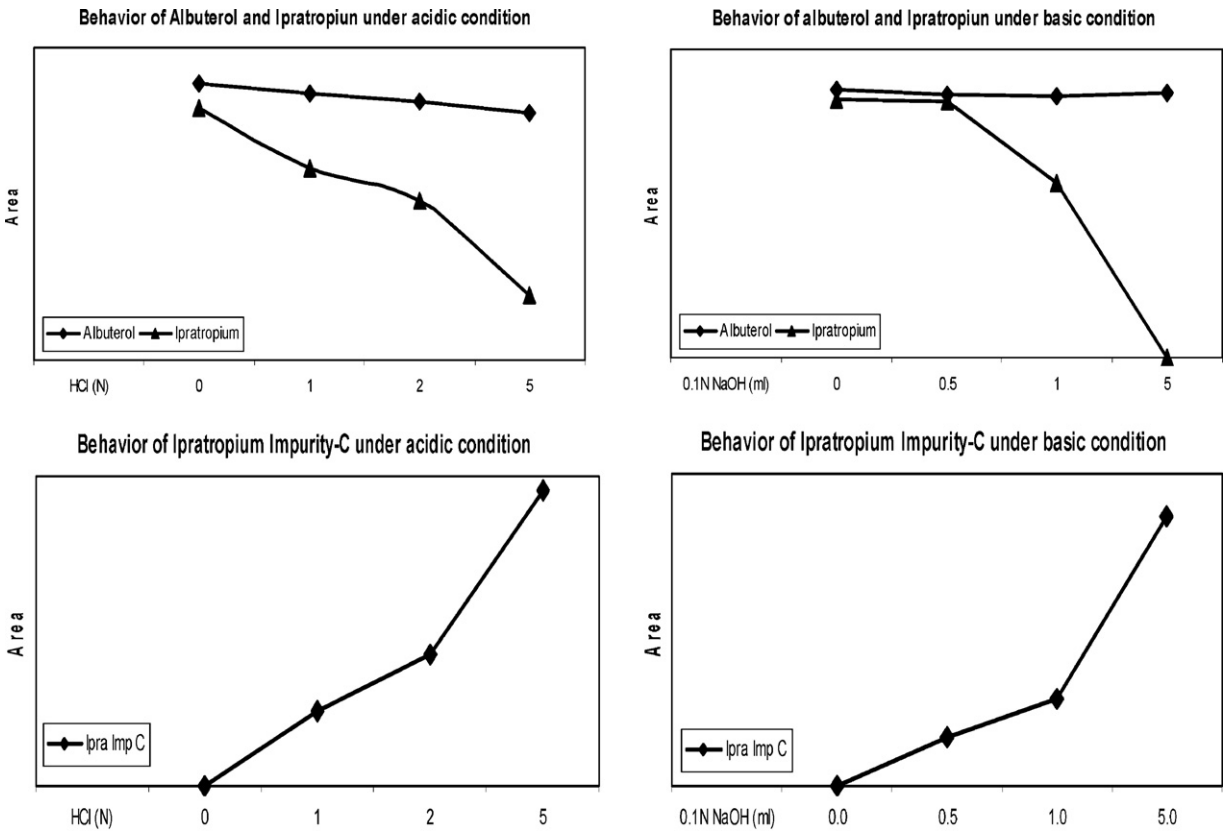


Fig. 7. Degradation trend under acidic and basic conditions with respect to albuterol sulfate, ipratropium bromide and ipratropium impurity-C as major degradent.

validation with unknown identities can be used as 1.) The experimental results revealed that approximately 95–105% recoveries were obtained for all the investigated related compounds. Therefore, based on the recovery data (Tables 6 and 7) the estimation of related compounds that are prescribed in this report has been demonstrated to be accurate for intended purpose and is adequate for routine analysis.

3.2.5. Method precision and ruggedness

ICH (International Conference on Harmonization of technical Requirements for Registration of Pharmaceuticals for Human Use) considers ruggedness as the method reproducibility and intermediate precision. The data obtained from linearity study was used for the evaluation of method ruggedness. The method reproducibility was determined from the %RSD of the recoveries obtained from nine samples prepared in triplicates at low (40%), middle (100%) and high (150%) concentration levels of typical impurity tolerance level of corresponding related known impurities. The intermediate precision was determined from the difference in the average recoveries and the difference in the %RSD of the recoveries among the three analysts. The results for all the tested compounds were listed in Tables 6 and 7 reveal that the method has good reproducibility and intermediate precision.

Table 5
Limit of quantification, detection, relative response factor (LOQ, LOD and RRF).

Impurities	LOQ ($\mu g\,ml^{-1}$)	LOD ($\mu g\,ml^{-1}$)	RRF
Albuterol sufate			
Albuterol	0.039	0.013	1.00
Impurity-J	0.053	0.017	1.65
Impurity-B	0.066	0.021	1.25
Impurity-C	0.076	0.025	1.38
Impurity-D	0.063	0.021	2.78
Impurity-H	0.053	0.018	1.12
Impurity-E	0.059	0.019	1.50
Impurity-G	0.094	0.031	1.51
Impurity-F	0.297	0.098	1.29
Impurity-I	0.054	0.018	2.05
Ipratropium bromide			
Ipratropium	0.031	0.010	1.00
Impurity-C	0.035	0.012	2.82
Impurity-D	0.042	0.014	4.75
Impurity-B	0.051	0.017	1.08
Impurity-E	0.049	0.016	1.30
Impurity-F	0.032	0.015	1.14

3.2.6. Robustness

In order to demonstrate the robustness of the method, system suitability parameters were verified by making deliberate change in chromatographic conditions, i.e. change in flow rate by $\pm 0.1\,ml\,min^{-1}$, change in pH of the buffer by ± 0.2 units, change in column oven temperature by $\pm 5\,^{\circ}C$ and change in organic composition of mobile phase by $\pm 2\%$ absolute. The sample spiked with all known impurities at impurity tolerance level was injected and the resolution among the impurities was monitored. The method was demonstrated to be robust over an acceptable working range of its HPLC operational conditions except the change in pH of buffer. Ipratropium impurities C and D shows the dramatic change in retention time with pH -variation affecting the resolution among the impurities. These two impurities move towards right with decreasing pH while towards left with increasing pH. Ipratropium impurity-D and albuterol impurity-C were co-eluted with decrease in pH while ipratropium impurity-D and albuterol impurity-B were co-eluted with increase in pH. Hence it was concluded that method is sensitive to mobile phase buffer pH.

Table 6
Precision and recovery.

Compound name	Average recovery (%)			Difference from analyst-1 (%)	
	Analyst-1[a]	Analyst-2[a]	Analyst-3[a]	Analyst-2	Analyst-3
Albuterol sufate					
Impurity-J	101.58	98.17	99.46	3.41	2.12
Impurity-B	98.89	100.05	101.06	1.16	2.17
Impurity-C	100.12	101.48	100.78	1.36	0.66
Impurity-D	100.21	100.03	102.46	0.18	2.25
Impurity-H	100.49	99.99	98.13	0.5	2.36
Impurity-E	99.33	98.99	97.89	0.34	1.44
Impurity-G	98.59	100.33	101.56	1.74	2.97
Impurity-F	103.88	103.58	100.49	0.30	3.39
Impurity-I	97.40	98.36	98.26	0.96	0.86
Ipratropium bromide					
Impurity-C	97.74	101.89	98.56	4.15	0.82
Impurity-D	100.23	99.26	102.12	0.97	1.89
Impurity-B	97.42	98.03	100.09	0.61	2.67
Impurity-E	100.64	99.39	97.77	1.25	2.87
Impurity-F	102.91	100.62	98.53	2.29	4.38

[a] $n = 6$.

Table 7
Precision and recovery.

Compound name ($n = 3$)	% RSD of recovery (%)			Difference from analyst-1	
	Analyst-1	Analyst-2	Analyst-3	Analyst-2	Analyst-3
Albuterol sufate					
Impurity-J	0.32	1.42	0.51	1.10	0.19
Impurity-B	2.12	2.83	1.16	0.71	0.96
Impurity-C	2.46	3.16	3.47	0.70	1.01
Impurity-D	3.56	2.78	1.33	0.78	2.23
Impurity-H	1.69	2.48	0.56	0.79	1.13
Impurity-E	1.92	3.15	0.46	1.23	1.46
Impurity-G	0.86	1.73	0.29	0.87	0.57
Impurity-F	0.97	1.91	2.23	0.94	1.26
Impurity-I	1.39	1.64	2.86	0.26	1.48
Ipratropium bromide					
Impurity-C	2.99	2.52	2.19	0.47	0.80
Impurity-D	1.18	2.29	1.92	1.11	0.74
Impurity-B	2.34	1.84	1.65	0.50	0.69
Impurity-E	0.96	2.06	2.16	1.10	1.20
Impurity-F	1.33	1.59	2.08	0.26	0.75

4. Conclusion

A stability study was carried out and an efficient HPLC method for the quantification of related substances of AS and IB in drug product was developed and validated. The results of the stress testing of the drug, undertaken according to the ICH guidelines, revealed that the degradation products were formed in hydrolytic (acid and base) conditions.

The kinetics of acid and base catalyzed degradation was also studied in 5N HCl and 0.1N NaOH and found to be first order reaction. The degradation rate increased linearly with increase in concentration of HCl (1–5N) and NaOH (0.5–5 ml).

Validation experiments provided proof that the HPLC analytical method is linear in the proposed working range as well as accurate, precise (repeatability and intermediate precision levels) and specific, being able to separate the main drug from its degradation products. The proposed method was also found to be robust with respect to flow rate, column oven temperature and composition of mobile phase. The method is very sensitive to pH of mobile phase. Due to these characteristics, the method has stability indicating properties being fit for its intended purpose, it may find application for the routine analysis of the related substances of AS and IB in AS + IB nasal solution.

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