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HPTLC-Densitometric and RP-HPLC Method Development and Validation for Determination of Salbutamol Sulphate, Bromhexine Hydrochloride and Etofylline in Tablet Dosage Forms

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Abstract

Different HPTLC-densitometric and RP-HPLC methods are developed for the simultaneous determination of salbutamol sulphate, bromhexine hydrochloride and etofylline in pharmaceutical tablets. HPTLC method was based on separation of all three drugs followed by densitometric measurements of their spots at 275 nm using CAMAG, TLC scanner III. The separation was carried out on Merck HPTLC aluminium plates of silica gel 60 F254, using acetone: methanol: formic acid (9:3:0.01) as mobile phase at room temperature (25 ± 2°C). The HPLC separation was carried out using a mobile phase consisting of 0.02 M ammonium acetate buffer: organic phase (where organic phase MeOH: ACN in ratio of 70:30) adjusted to pH 4.5 with gradient elution. The column used was Waters Spherisorb®C_{18} bonded with 5 µm (4.6 x 250 mm) with a flow rate of 1 mL min^{-1} and UV detection at 275 nm simultaneously. The mean recovery ofboth HPTLC and HPLC methods were found to be within 99.44 to 99.85 % w/w. Both HPTLC-densitometric and RP-HPLC methods were statistically validated and can be used for analysis of combined dose tablet formulation containing salbutamol sulphate, bromhexine hydrochloride and etofylline.

Keywords: Salbutamol sulphate; Bromhexine hydrochloride; Etofylline; HPTLC; HPLC; UV detection

Abbreviations: BH- Bromhexine hydrochloride; SS- Salbutamol Sulphate; ET- Etofylline; HPLC- High Performance Liquid Chromatography; HPTLC- High Performance Thin Layer Chromatography; GAA- Glacial acetic acid

Introduction

Salbutamol sulphate (SS), as shown in Figure 1 chemically known as bis [(1RS)-2-[(1, 1-dimethylethyl) amino]-1-[4-hydroxy-3-(hydroxymethyl) phenyl] ethanol] sulphate, is beta adenocepter agonist. It is used for the relief of bronchospasm in condition such as asthma [1,2] and chronic obstructive pulmonary disease [3,4]. The smooth muscles are relaxed by the increase in the intracellular cyclic adenosine monophosphate [5-7]. Bromhexine hydrochloride (BH)as shown in Figure 1, N-(2-amino-3, 5-dibromobenzyl)-N methyl cychlohexanamine hydrochloride, is an expectorant use in the treatment of various respiratory disorders. Etofylline (ET) as shown in Figure 1, 7-(2-hydroxyethyl)-1, 3-dimethyl-3, 7-dihydro-1, 4-purine-2, 6-dione, is a xanthine bronchodilator used for the treatment of respiratory diseases and asthma in combination with SS. SS [8], BH [8] and ET [8] are official in BP. Official methods involve determination of SS, BH and ET using Potentiometry.SS, BH and ET as component of a multi ingredient formulation and has been reported to be estimated by some spectroscopic and HPLC methods either single or in combination simultaneously [9-15]. One spectroscopic method has been reported for the determination of SS along with BH and ET in combined dosage forms [16]. The tertiary combination SS, BH and ET, is not yet official in any pharmacopoeia. As par literature, no RP-HPLC and HPLC methods could be traced for the analysis of SS, BH and ET in their combined dosage forms. Therefore simple, rapid, economical and reliable RP-HPLC method for estimation of these drugs in mixture seemed to be necessary. All the analytical and validation procedures followed in the present study were as per ICH guidelines [17,18].

Experimental

Instruments

Camag HPTLC system equipped with a sample applicator Linomat 5 TLC Scanner III, Reprostar and Wincats 4.02 integration software (Switzerland), twin trough glass development chamber, Ultrasonic cleaner (Sterylmedi-equip systems) and water purification system ELIX 03 (MILLIPORE, USA) were used during study.HPLC system of Waters (Milford, USA) composed of 515 series pumps combined with Waters 2707 autosampler along with Waters PDA 2998 series photodiode array detector set at wavelength range 190-800 nm with column from Waters Spherisorb®C_{18} bonded with 5 µm (4.6 x 250 mm) coupled with EMPOWER-2 software recording and processing of chromatographic data was used.

Chemicals and reagents

SS, BH and ET were supplied as gift sample from FDC Ltd. (Mumbai, India) and marketed formulation of Eto-salbetol-10 (Batch Number–BTK0017, Kare Labs Pvt. Ltd., Goa, India) was procured from the local drug store, Moga, Punjab. Acetonitrile, methanol, water, ammonium acetate and glacial acetic acid (GAA) were purchased from Rankem (New Delhi, India). All these chemicals and solvents

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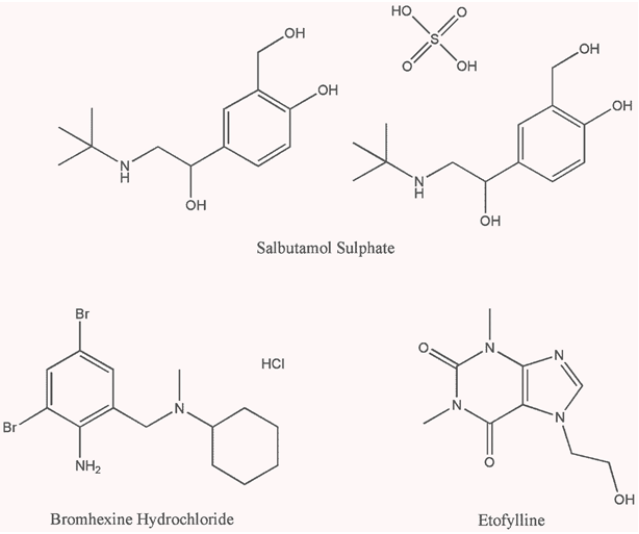

Figure 1: Structure of SS, ET and BH

were of HPLC grade and were used without any further purification. All the required solutions were prepared in HPLC grade water. HPLC grade water was obtained from water purification systems ELIX 03 (MILLIPORE, USA). Unless otherwise specified, all solutions were filtered through a 0.2 µm Ultipor® N66® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) prior to use. Pre-coated silica gel 60 F_{254} TLC aluminium backed plates (20 x 20 cm, 0.2 mm thick) were purchased from E. Merck Ltd. (Germany).

Chromatographic conditions

HPTLC plates were developed with acetone**:** MeOH**:** formic acid (9**:** 3**:** 0.01) as mobile phase. Chromatographic plate was developed up to the top (over a distance of 8 cm) in the usual ascending way. Chromatographic tank was saturated with mobilephase in the usual manner. After elution, plate was air dried and scanned at 275 nm as under the described instrumental parameters. Samples were spotted as a band of 8 mm width on pre-coated silica gel aluminium backed plate 60F-254 with 200 µm thickness. Application rate of 80 nl s^{-1} was employed and space between bands was 10 mm. Linear ascending development was carried out in a twin trough glass chamber previously saturated with mobile phase for 20 min. Densitometric scanning was performed on camag TLC scanner III in the absorbance/reflectance mode.For HPLC, mobile phase consisting 0.02 M ammonium acetate buffer**:** organic phase (where organic phase MeOH**:** ACN in ratio of 70**:**30) adjusted to pH 4.5with 0.6% GAA was prepared, filtered through 0.45 µm membrane filter and degassed by an ultrasonic bath before each use. Best resolution and sensitivity of the method was obtained for SS, BH and ET at 275 nm at a flow rate of 1.0 mL min^{-1} with gradient elution. The column was equilibrated for at least 30 min with the mobile phase flowing through the system. Each solution was injected in triplicate.

Preparation of standard stock solutions

Stock solutions of SS, BH and ET (100 µg mL^{-1}) were freshly prepared by dissolving in MeOH individually and stored at 2–8°C until used. The standard solutions were prepared by dilution of the stock standard solutions with MeOH (for HPTLC method) or with the mobile phase composition (for HPLC method) to obtain 10 µg mL^{-1}. These solutions were used for optimization of proposed methods to reach the calibration curve for specified each method.Commercial formulations containing tablets of Eto-Salbetol (Batch Number-BTK0017, Kare Labs Pvt. Ltd., Goa , India), which were labeled to contain 2 mg of SS, 8 mg of BH and 200 mg of ET per tablets were used for the study.

Sample application

In HPTLC, the samples were spotted as a band of 8 mm width on pre-coated silica gel aluminium backed plate 60F-254 with 200 µm thickness. Application rate of 80 nl $s^{-1}$was employed and space between bands was 10 mm. Linear ascending development was carried out in a twin trough glass chamber previously saturated with mobile phase for 20 min. Densitometric scanning was performed on camag TLC scanner III in the absorbance/reflectance mode.In HPLC, the samples were filtered through a 0.2 µm membrane filter using syringe filter holder. An aliquot of 20 µl was injected with the help of Waters 2707 auto sampler.

Optimization of chromatographic conditions

HPTLC method: Chromatographic separations are significantly affected by mobile phase conditions, such as type and composition of organic modifiers therefore before selecting proper chromatographic conditions, numbers of preliminary trials were conducted with different combinations of different organic solvents, compositions, to obtain satisfactory retention factor, resolution, and other chromatographic parameters. From those experiments mobile phase combination of acetone**:** MeOH**:** formic acid (9**:** 3**:** 0.01) was found to be most suitable. Best resolution and sensitivity of the method for SS, BH and ET was detected at 275 nm. Typical chromatogram with optimized condition gave sharp and resolved peaks with retention factor of 0.51, 0.76 and 0.85 for SS, ET and BH respectively. The developed chromatogram of SS, BH and ET are shown in (Figure 2).

HPLC method: In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase composition varied only in organic phase (60% v/v) keeping aqueous phase constant (40 % v/v). Organic phase composition varied in different ratio of MeOH**:** ACN such as 50**:**50, 60**:**40 and 70**:**30 with fixed flow rate of 1.0 mL min^{-1} (ii) Change in pH such as 4.0, 4.5, and 5.0 with mobile phase composition 40**:**60 of 0.02 M ammonium acetate buffer**:** organic phase (MeOH**:** ACN in ratio of 70**:**30) (iii) Flow rate was varied such as 0.8, 1.0, and 1.2 mL $min^{-1}$with mobile phase composition in ratio of 40**:**60 of 0.02 M ammonium acetate buffer**:** organic phase (MeOH**:** ACN in ratio of 70**:**30) and pH maintained at pH 4.5. Moreover, effects of three factors i.e. mobile phase composition, pH and flow rate were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc. After various trials of different chromatographic parameters, optimum mobile phase was found to be 0.02 M ammonium acetate buffer**:** organic phase (where organic phase MeOH**:** ACN in ratio of 70**:**30) adjusted to pH 4.5 with gradient elutionat flow rate of 1 mL min^{-1}. Best resolution and sensitivity of the method was obtained for SS, BH and ET at 275 nm. Typical chromatogram with optimized condition gives sharp and symmetric peak with retention time of 2.8 min, 3.8 min and 13.2 min for ET, SS and BH respectively. Developed chromatogram of SS, BH and ET are shown in (Figure 3).

Validation of optimized method: After the chromatographic method had been optimized and developed, it must be validated; analytical method validation shows that the characteristics of the method satisfy the requirements of the application province. The

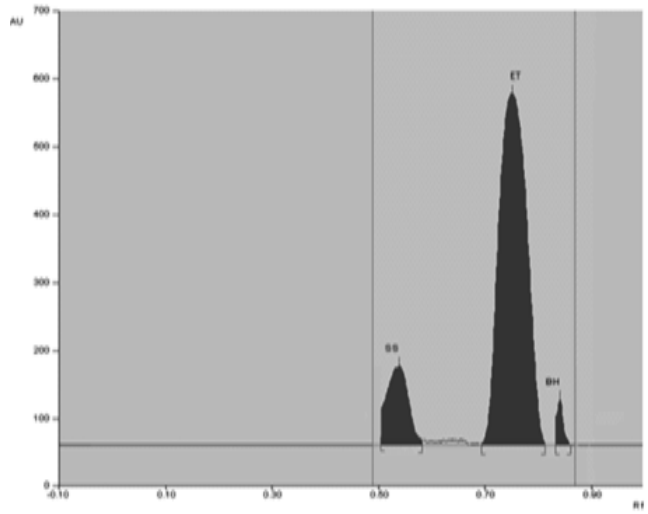

Figure 2: Chromatographic peaks of SS, ET and BH by optimized HPTLC method

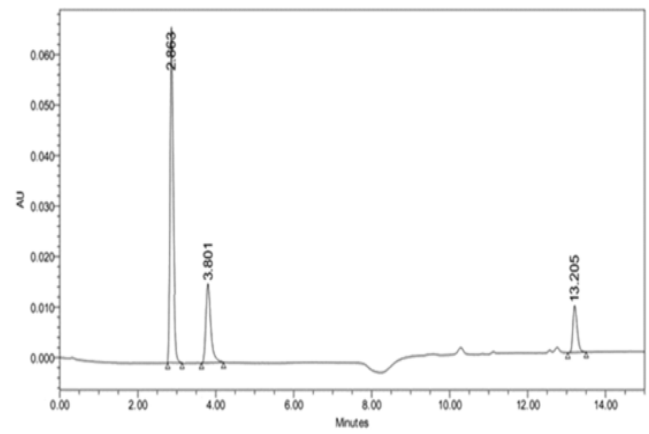

Figure 3: Chromatographic peaks of ET, SS and BH for optimized HPLC method.

proposed method was validated in the lime light of ICH guidelines for linearity, range, accuracy, precision, and limit of detection, limit of quantification, sensitivity, and recovery. Consequently, the following were performed.

Linearity: Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity was checked by diluting standard stock solution at six different concentrations. The calibration plot (peak area ratio of SS, BH and ET) was generated by replicate analysis (n=3) at all concentration levels. Linearity was done by six different concentrations of SS, BH and ET in triplicate and calibration curve was plotted in specified range of 1-6 µg spot^{-1} of SS and BH and 0.5-3.0 µg spot^{-1} of ET for HPTLC and 1-32 µgmL^{-1} of SS and BH and 0.5-16 µgmL^{-1} of ET for HPLC respectively. Calibration curve was plotted by replicate analysis at all concentration levels and linear relationship was evaluated using the least square method within Graph Pad Prism 5.01.0.

Precision and accuracy: The precision of the method was confirmed by repeatability and intermediate precision. It expresses the closeness of arrangement between a series of arrangements obtained from multiple sampling of the same homogeneous sample. The intermediate precision of the method was confirmed by intraday and interday analysis was repeated three times in the same day and on three successive days. The absorbance was determined and % Relative Standard Deviation (RSD) also calculated.Accuracy expresses the arrangement between measured value and real value. To check the accuracy of the developed method and to study the interference of formulation excipients, analytical recovery experiments were carried out by using standard addition method in three different concentrations that is 80%, 100% and 120% of the standard drug. From the total amount of drug found, the percentage recovery was calculated. This procedure was repeated for three times for each concentration. Accuracy precision for the developed method was measured in terms of % RSD.

Specificity: This was ascertained by analyzing the R_f values and spectra pattern of standard drug and marketed formulation. The peak purity of sample was determined by spectral comparison of sample peak at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions.

Sensitivity: Sensitivity of both the methods was determined by calculating LOB, Limit of detection (LOD) and Limit of Quantification (LOQ) using following equation:

LOB =1.645 σ/S

LOD =3.3 σ/S

LOQ =10 σ/S

Where σ= Standard deviation of the response; S = Slope of the calibration curve

Robustness: Robustness is small but deliberate change in samples under a variety of normal test conditions such as variation in solvent composition and wavelength. In present study, determination of SS, BH and ET were carried out by using change in mobile phase composition, mobile phase volume, and saturation time, activation time of pre-coated plates for HPTLC and change in mobile phase, Flow rate, pH, and detection wavelength for HPLC. The deviations in the results of peak area were expressed as % RSD.

Percentage purity: Twenty tablets were accurately powdered and weighed equivalent to 2 mg of SS, 8 mg of BH and 200 mg of ET per tablets were used for the study and diluted up to 20 ml with methanol. This dilution was used for spotting on stationary phase of method. Presence of SS, BH and ET in commercial formulations was confirmed by comparing its R_f, overlay (Figure 4). Purity of band of SS, BH and ET in sample (Figure 5) was determined by HPLC chromatogram by comparing UV absorption spectra at the start, middle, and end position of band with that of reference.

Results and Discussion

For the separation of non-polar and/or non-ionic substances normal phase chromatography can be used, while separation of non-ionic as well as ion forming non-polar to medium polar substances reversedphase chromatography (C8,C18) can be used. Thus, SS, BH and ET can be satisfactorily separated by reversed phase chromatography. Majority of the ionizable pharmaceutical compounds can be very well separated on octadecylsilane reversed phase columns. Hence, octadecylsilane was selected. Sensitive, Rapid and novel HPLC method

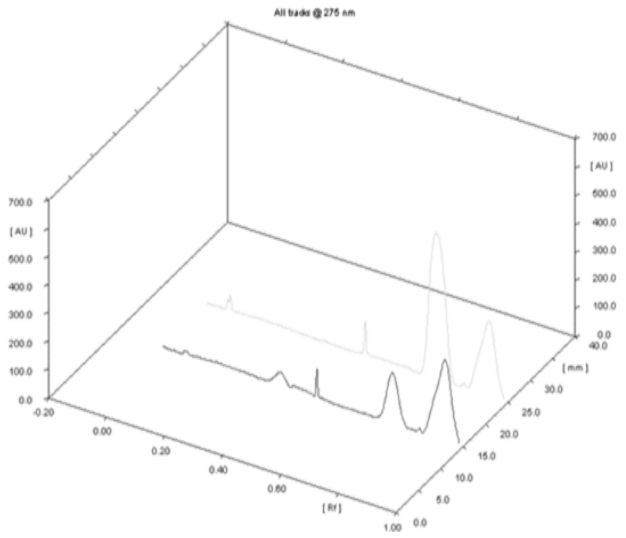

Figure 4: Overlay spectra of standard and marketed formulation for tertiary mixture of SS, ET and BH.

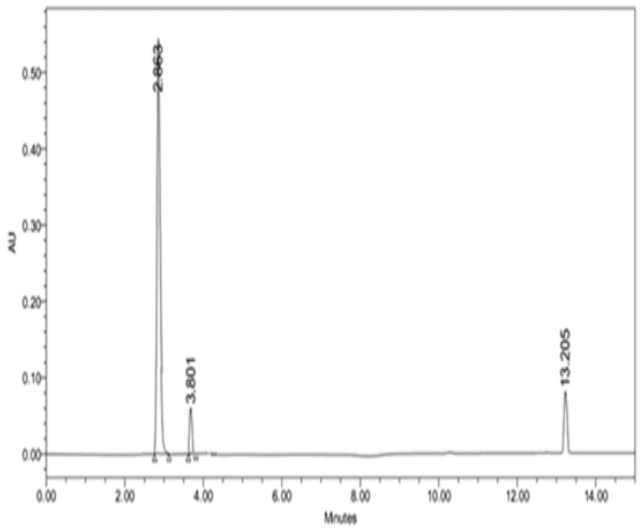

Figure 5: Chromatographic peaks of ET, SS and BH in presence of excipients in tablet dosage form.

for determination of ternary mixture of drugs was optimized and validated.

Optimization of chromatographic conditions

Effect of mobile phase pH: With the aim of the optimization of mobile phase pH (4, 4.5 and 5), the remaining two factors were kept constant, i.e. mobile phase composition (MeOH: ACN; 70:30, v/v) and flow rate of 1.0 mL min^{-1}. Observed chromatographic responses were plotted against respective pH. As shown in the Figure 6, retention time increases with the increase in pH while asymmetry decreases. The number of theoretical plates as well as resolution of SS, BH and ET was maximum at pH 4.5. Looking at the importance of the different chromatographic parameters, pH 4.5 was found to be optimum. The dissociation constant (pKa) of SS is 10.3, BH is 8.34 and ET is 13.39 at (21–24°C). The asymmetry value decreases with increase in pH. At lower pH the ET will be carried out faster with mobile phase, however, due to higher unionized species. This result in tailing and hence increase in asymmetry value at lower pH. At higher pH value, the ionized hydrophilic species are not much portioned with stationary phase and hence gives symmetric peak. The resolution was poor at pH 4.0, but was highest at pH 5.0. Similarly, the plate number (highest for higher retention time and smallest peak width) was highest at pH 5.0. Thus, the best chromatographic separation was achieved at pH 4.5, and hence was considered to be optimum.

Effect of mobile phase composition: The effect of mobile phase composition (i.e. ratio of MeOH: ACN was studied at 50:50, 60:40 and 70:30 v/v levels and the flow rate of 1.0 mL min^{-1} is shown in Figure 7. Minimum retention times of SS, BH and EZ were obtained at 70:30 v/v level, which makes the method rapid, a one of the most desirable criteria. Though retention time was shorter, satisfactory resolution and asymmetry values were achieved. An adequate theoretical plates (~12000) is indicative of a good column performance As can be seen from Figure 7, the asymmetry was >1.7 at 50:50 and 60:40 v/v which indicates tailing of the peaks, but was <1.9 at 70:30, v/v.Similar findings were observed in the present study up to 50:50, v/v composition, but further increase in ACN content resulted in increased retention time in SS and BH and decreases for BH. However, at 70:30, v/v composition, proper balance was attained and resulted in least retention time. The least asymmetry at 70:30 compared to other two compositions can be explained on the same basis. Plate number increased with increase in ACN composition in mobile phase. However, the asymmetry value at 60:40 v/v was higher than that of at 70:30 v/v. These suggest that the increased plates at 70:30, v/v was due to higher retention time value (even though it had greater peak width due to tailing). Further, acceptable resolution (>2) was achieved at 70:30, v/v composition and so was considered to be optimum.

Effect of mobile phase flow rate: From Figure 8, it can be observed that theoretical plates were highest at flow rate of 1.0 mL min^{-1} with asymmetry of less than 1.2. The change in flow rate had no significant effect on resolution. While retention time decrease as the flow rate increased with poor plates and higher asymmetry. Further, acceptable resolution (>2) with plates was achieved at 1.0 mL min^{-1} and so was considered to be optimum.

Proposed chromatographic method: The developed chromatogram SS, BH and ET gave sharp UV absorbance peak at275 nm with mobile phase containing acetone: MeOH: formic acid (9:3:0.01) with R_f values 0.51, 0.76 for SS, ET and 0.85 for BH respectively for HPTLC. Looking at the different chromatographic parameters during the method development, the finally recommended mobile phase consisted of MeOH: ACN 0.02 M of 70:30, v/v adjusted to pH 4.5. The best resolution and sensitivity of the method was obtained at 275 nm and mobile phase flow rate of 1 mL min^{-1}. Typical chromatogram at the optimized condition gave sharp and symmetric peak with retention time of 2.8, 3.8 and 13.2 min for ET, SSand BH respectively.

Validation of the proposed method

Calibration curve (linearity): Calibration curve (peak area ratio of SS, BH and ETconcentration) in mobile phase. The chromatographic responses were found to be linear over an analytical range of 1-6 µg $spot^{-1}$ of SS and BH and 0.5-3.0 µg $spot^{-1}$ of ET with a correlation coefficient of 0.998 for SS and 0.997 for BH and ET respectively for HPTLC and Relationship between the concentration of standard solutions and peak response was linear within the concentration range of 1-32 µgmL^{-1} of SS and BH and 0.5-16 µg$mL^{-1}$of ET with correlation coefficient equals

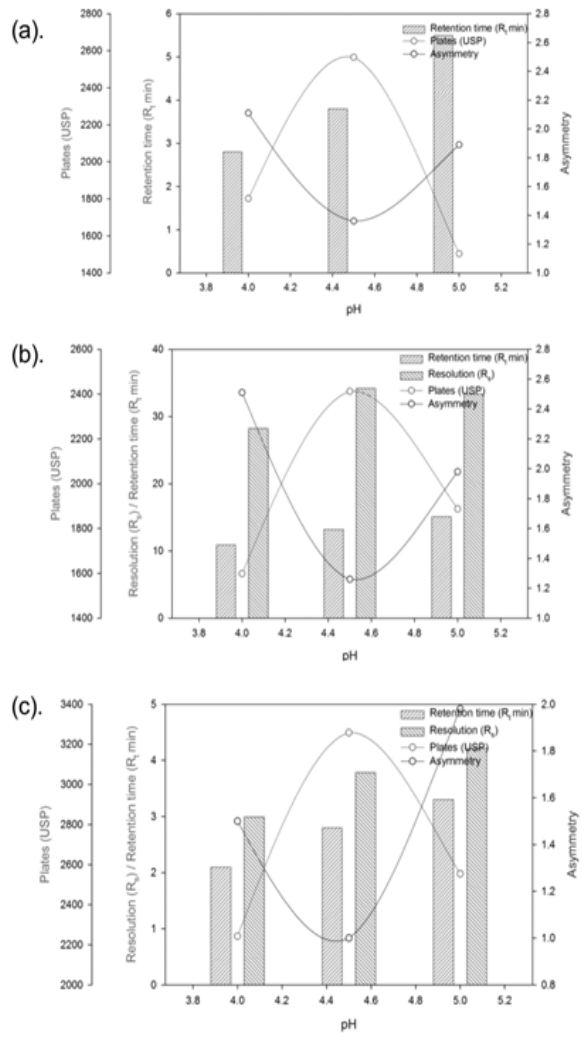

Figure 6: Effect of mobile phase pH (4, 4.5 and 5) at mobile phase composition of 70:30 (MeOH:ACN v/v) and flow rate of 1.0 mL min^{-1}; (a) for S.S. (b) for B.H. and (c) for E.T.

0.999 for HPLC and found to be quite satisfactory and reproducible with time. The linear regression equation was calculated by the least squares method using Graph Pad Prism 5.01.0 program. The variance of slope and intercept were reported in Table 1 and 2. This shows that the intercept is not significantly different from zero, indicating no interference in the estimations. Further the slope and intercept were within the confidence interval.

Accuracy and precision: Accuracy data in the present study is performed by three different combination of marketed formulations ranged from 98.75 to 99.99% for HPTLC and 98.75 to 99.96 for HPLC indicates that there was no interference from excipients components. Interday as well as intraday replicates of SS, BH and ET gave an RSD below 2.0 (should be less than 2 according to Center for Drug Evaluation and Research (CDER) guidance for analytical method validation), revealed that the proposed method is highly precise. Accuracy of the method was evaluated by using t-test at four concentration levels including the lowest quantifiable level. The t-values obtained for different spiked amount of SS, BH and ET. The t-value required for significance at 5% level at 5 degrees of freedom is 2.57, and the obtained values were well below this value. Thus no significant difference was observed between the amounts of drug added and recovered. Overall, the data summarized in Table 3 and 4, enables the conclusion that an excellent accuracy and high precision was obtained.

Robustness: The % RSD was found to be less than 1% for the method, which support the effectiveness and usefulness of the method respectively and percentage purity of SS, BH and ET were found to be 99.41 ± 1.09 % w/w, 98.36 ± 0.33 % w/w and 98.89 ± 0.04 % w/w respectively for HPTLC and 99.93 ± 1.33% w/w, 99.48 ± 0.57% w/w and 97.34 ± 0.02% w/w respectively shown in table for HPLC. Robustness studies for both methods are shown in Tables 5 and 6.

Sensitivity: The LOD and LOQ for SS, BH and ET were found to be 100, 200 and 10 and 330, 660 and 33 $ngmL^{-1}$ for HPTLC and 150, 200 and 10 and 495, 660 and 49 for HPLC respectively. The present method was selective enough to and can be applied for simultaneously analyze SS, BH and ET in marketed pharmaceutical formulation, its sensitivity was found to be adequate for assay and dissolution test of tablets for the estimation of title drugs in their commercial samples.

Specificity: Specificity is the extent to which the procedure applies to analyte of interest and is checked by examining the formulation samples for any interfering peaks. The specificity of the method was

Parameters	SS	BH	ET
Absorption maxima (nm)	275	275	275
Linearity range (µg $spot^{-1}$)	1-6	1-6	0.5-3.0
Coefficient of determination (r^2)	0.998	0.997	0.998
Correlation coefficient (r)	0.999	0.999	0.999
Regression equation (Y^a)	Y=488.2x+754.7	Y=292.3x+116.7	Y=5291x+3476
Slope (m)	488.2	292.3	5291
$t_{cal}{}^m$	1.3234	1.2857	0.1323
Confidence interval^m	488.2 ± 21.67	292.3 ± 3.99	5291 ± 100.62
Intercept (c)	754.7	116.7	3476
$t_{cal}{}^m$	0.5249	0.6368	1.4692
Confidence interval^m	754.7 ± 59.23	116.7 ± 19.62	3476 ± 103.52
Limit of detection LOD, (ng)	100	200	10
Limit of quantification LOQ (ng)	330	660	33
Precision, Intra-day (%RSD) Inter-day (%RSD)	0.79 0.53	0.94 1.21	0.31 0.26

Table 1: Validation parameters of HPTLC method for SS, BH and ET

Parameters	SS	BH	ET
Absorption maxima (nm)	275	275	275
Linearity range (µg mL^{-1})	1-32	1-32	0.5-16
Coefficient of determination (r^2)	0.997	0.998	0.998
Correlation coefficient (r)	0.999	0.999	0.999
Regression equation (Y^a)	Y=4253x-2508	Y=16462x-403.9	Y=25609x-3524
Slope (m)	4253	16462	25609
t_{cal}^m	1.5241	1.2872	0.5341
Confidence interval^m	4253 ± 91.67	16462 ± 59.77	25609 ± 274.62
Intercept (c)	-2508	-403.9	-3524
t_{cal}^m	0.0007	0.0162	0.0100
Confidence interval^m	-2508 ± 649.06	-403.9 ± 544.72	-3524 ± 923.37
LOB (µg mL^{-1})	0.0455	0.0606	0.0045
LOD (µg mL^{-1})	0.1500	0.2000	0.0150
LOQ (µg mL^{-1})	0.4950	0.6600	0.0495
Precision, Intra-day (%RSD) Inter-day (%RSD)	0.72 1.17	0.45 0.89	0.30 0.52

Table 2: Validation parameters for RP-HPLC method of SS, BH and ET.

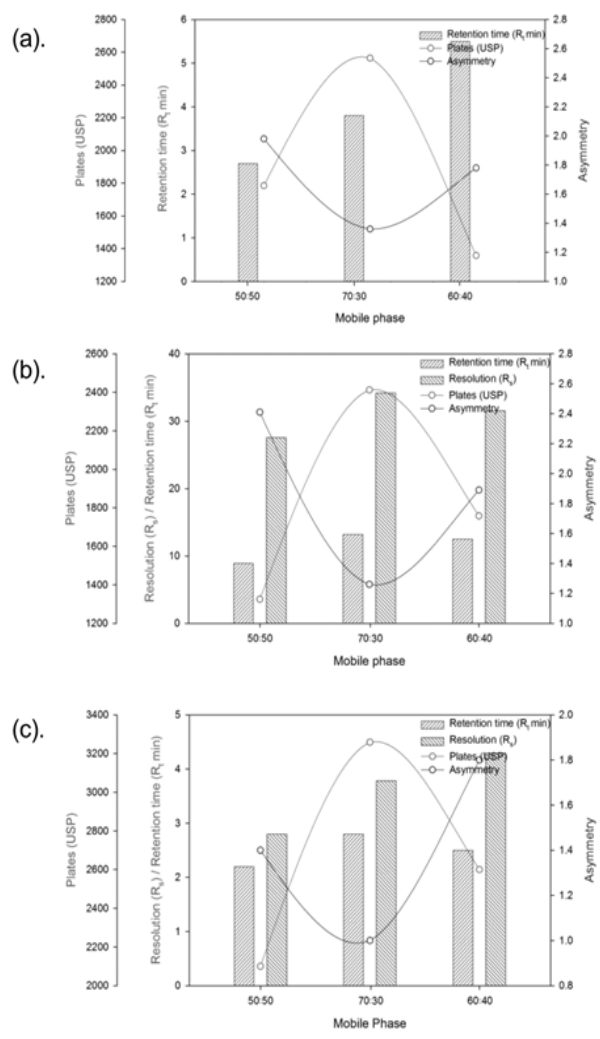

Figure 7: Effect of mobile phase composition (50:50,70:30 and 60:40 MeOH:ACN v/v) at mobile phase ph 4.5 and flow rate of 1.0 mL min^{-1}; (a) for S.S. (b) for B.H. and (c) for E.T.

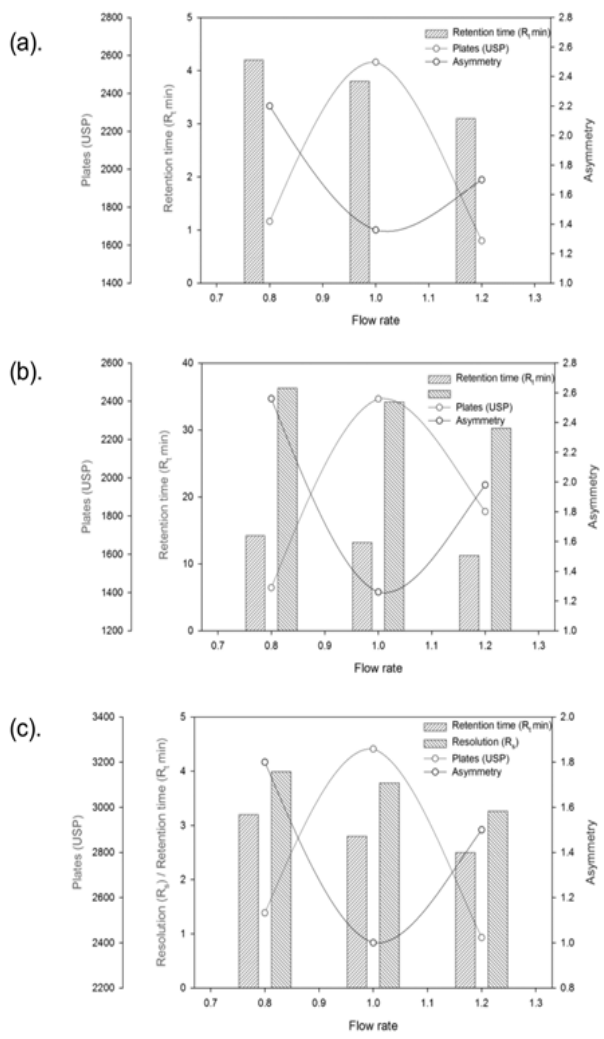

Figure 8: Effect of flow rate (0.8, 1.0, and 1.2 mL min^{-1}) at mobile phase pH 4.5 and mobile phase composition 70:30 MeOH:ACN v/v (a) for S.S. (b) for B.H. and (c) for E.T.

evaluated with regard to interference due to presence of excipients. The excipients used in formulation did not interfere with the SS, BH and ET peaks and thus the method is specific.

System suitability: System suitability tests, an integral part of a chromatographic analysis are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis [24]. A system suitability test according to USP was performed on the chromatograms obtained from standard and test solutions to check different above mentioned parameters and the results obtained from six replicate injections of the standard solution are summarized in the Table 7.

Formulation analysis: The proposed methods were applied to the determination of SS, BH and ET in commercial tablets. Satisfactory results were obtained for tertiary mixture of drugs and were in good agreement with the label claims (Table 8). Moreover, to check the validity of the proposed methods, the standard addition method was applied by adding SS, BH and ET to the previously analyzed tablets. The recovery of each drug was calculated by comparing the concentration obtained from the spiked mixtures with those of the pure drug. The results of analysis of the commercial tablets and the recovery study (standard addition method) of three drugs (Tables 3 and 4) suggested that there is no interference from any excipients, which are present in tablets.

Statistical analysis: A statistical comparison of the results was performed with regard to accuracy and precision using Student's t-test at a 95% confidence level (Tables 3 and 4). The t-values obtained for different spiked amount of SS, BH and ET. The t-value required for significance at 5% level at 5 degrees of freedom is 2.57, and the obtained values were well below this value. Thus no significant difference between the proposed was observed between the amounts of drug added and recovered with regard to accuracy and precision. The descriptive stastical analyses were also applied on the HPTLC and HPLC methods (Tables 9 and 10) which further confirm the sensitivity and accuracy of the proposed method for the estimation of SS, BH and ET in commercial formulations.

Conclusion

Accurate results for determination of individual drug in combination of single pharmaceutical dosages form by applying HPTLC and HPLC method were achieved. The proposed HPTLC and HPLC methods provide simple, accurate, and reproducible quantitative analysis for simultaneous determination SS, BH and ET in tablets. The HPLC method is more rapid in terms of sensitivity and accuracy than HPTLC. The proposed validated methods are good alternative for the simultaneous estimation of tertiary mixture in pharmaceutical formulation. This method has been found to be suitable for the successful simultaneous estimation of the SS, BH and ET in the formulation. This method can also be applied for the estimation of these drugs in other combination or can also be used for the estimation of these drugs individually.

References

Parameters	SS	BH	ET
Absorption maxima (nm)	275	275	275
Linearity range (µg spot^{-1})	1-6	1-6	0.5-3.0
Coefficient of determination (r^2)	0.998	0.997	0.998
Correlation coefficient (r)	0.999	0.999	0.999
Regression equation (Y^a)	Y=488.2x+754.7	Y=292.3x+116.7	Y=5291x+3476
Slope (m)	488.2	292.3	5291
$t_{cal}{}^m$	1.3234	1.2857	0.1323
Confidence interval^m	488.2 ± 21.67	292.3 ± 3.99	5291 ± 100.62
Intercept (c)	754.7	116.7	3476
$t_{cal}{}^m$	0.5249	0.6368	1.4692
Confidence interval^m	754.7 ± 59.23	116.7 ± 19.62	3476 ± 103.52
Limit of detection LOD, (ng)	100	200	10
Limit of quantification LOQ (ng)	330	660	33
Precision, Intra-day (%RSD) Inter-day (%RSD)	0.79 0.53	0.94 1.21	0.31 0.26

Table 3: Results of recovery study of SS, BH and ET by HPTLC Method.

Amount Spike			Amount found			SD			%RSD			Recovery			t_{cal}			CI		
SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET	SS	BH	ET
Intra-day (n=3)																				
1.6	6.4	160	1.59	6.39	159.54	0.0058	0.0153	0.7427	0.3616	0.2389	0.4655	99.79	99.90	99.71	1.00	0.76	1.06	0.0086	0.0227	1.1045
2.0	8.0	200	1.99	7.99	199.39	0.0100	0.0153	0.7396	0.5025	0.1913	0.3709	99.50	99.83	99.69	1.72	1.51	1.43	0.0149	0.0227	1.0999
2.4	9.6	240	2.38	9.59	239.58	0.0153	0.0100	0.8145	0.6409	0.1043	0.3400	99.31	99.90	99.82	1.88	1.73	0.90	0.0227	0.0149	1.2112
Inter-day (n=6)																				
1.6	6.4	160	1.59	6.38	159.54	0.0141	0.0207	0.9247	0.89	0.3236	0.5796	99.38	99.74	99.71	1.72	1.97	1.21	0.0210	0.0307	1.3752
2.0	8.0	200	1.98	7.99	199.48	0.0137	0.0137	1.3012	0.69	0.1711	0.6523	99.17	99.83	99.74	2.96	2.39	0.97	0.0203	0.0203	1.9351
2.4	9.6	240	2.38	9.57	239.83	0.0147	0.0279	1.0394	0.62	0.2907	0.4334	99.51	99.88	99.93	1.93	1.02	0.40	0.0219	0.0414	1.5458

Table 4: Results of recovery study of SS, BH and ET by HPLC Method.

Parameters	Alterations	%RSD for Retention factor (n=6)			%RSD for Area (n=6)			%RSD for Assay (n=6)		
		SS	BH	ET	SS	BH	ET	SS	BH	ET
Mobile phase composition	-2%	0.1121	0.2622	0.2739	0.2969	0.2787	0.2753	0.1462	0.2749	0.3119
	Normal	0.1289	0.1664	0.2642	0.3180	0.2761	0.2891	0.1841	0.3188	0.3992
	+2%	0.1379	0.1785	0.1989	0.2984	0.2835	0.2342	0.2101	0.3218	0.3861
Mobile phase volume	-2.0unit	0.3121	0.2963	0.4561	0.1623	0.3429	0.3041	0.2572	0.3176	0.4172
	Normal	0.3462	0.2784	0.6439	0.1343	0.4119	0.2801	0.3442	0.3219	0.4974
	+2.0unit	0.3429	0.3072	0.4534	0.1587	0.3741	0.2420	0.3596	0.4281	0.4990
Saturation time	-5.0unit	0.2943	0.3257	0.4422	0.2039	0.4752	0.2991	0.3186	0.4376	0.5469
	Normal	0.2845	0.3724	0.4753	0.2205	0.4969	0.3527	0.2792	0.4810	0.5670
	+5.0unit	0.3108	0.3572	0.4631	0.2265	0.5264	0.4124	0.3126	0.5292	0.5911
Activation time of pre-coated plates	-2.0unit	0.2191	0.2652	0.1540	0.1872	0.2719	0.1041	0.2389	0.2110	0.4002
	Normal	0.2349	0.2743	0.2439	0.1943	0.3287	0.2671	0.3219	0.2397	0.4784
	+2.0unit	0.2429	0.3017	0.3576	0.2580	0.3551	0.2299	0.3109	0.2639	0.4892
Detection wavelength	-0.4unit	0.0132	0.4873	0.2682	0.4129	0.3758	0.4944	0.4480	0.4632	0.3481
	Normal	0.1201	0.5621	0.2872	0.4483	0.3432	0.4130	0.5702	0.4671	0.3630
	+0.4unit	0.2091	0.5892	0.2249	0.4882	0.3774	0.3873	0.4165	0.4610	0.3979

Table 5: Robustness parameter of SS, BH and ET studied for HPTLC method.

Parameters	Alterations	%RSD for Retention time (n=6)			%RSD for Area (n=6)			%RSD for Assay (n=6)		
		SS	BH	ET	SS	BH	ET	SS	BH	ET
Mobile phase	-2%	0.2143	0.2812	0.2018	0.2942	0.3782	0.4871	0.2431	0.3872	0.4321
	Normal	0.2452	0.1466	0.2167	0.2180	0.3764	0.4813	0.2761	0.4286	0.4982
	+2%	0.1462	0.1676	0.1972	0.2943	0.3842	0.4319	0.3108	0.4202	0.4874
Flow rate	-10%	0.3113	0.4961	0.4563	0.1278	0.4411	0.3042	0.4532	0.4022	0.5487
	Normal	0.3871	0.5786	0.6431	0.1132	0.5039	0.1892	0.5431	0.4135	0.5982
	+10%	0.3217	0.6071	0.4532	0.1484	0.4988	0.2791	0.4596	0.5014	0.4983
pH	-0.2unit	0.3924	0.4254	0.5432	0.2097	0.4373	0.3921	0.3216	0.6165	0.6732
	Normal	0.3854	0.4711	0.5761	0.2243	0.4828	0.4506	0.2988	0.6746	0.6178
	+0.2unit	0.4116	0.4582	0.5629	0.2198	0.5498	0.5106	0.3116	0.5153	0.6988
Detection wavelength	-0.2unit	0.3132	0.6464	0.2678	0.4173	0.5876	0.2984	0.5473	0.3628	0.2756
	Normal	0.3201	0.6919	0.2891	0.4442	0.5342	0.2135	0.6759	0.3684	0.2431
	+0.2unit	0.3091	0.5867	0.2289	0.4892	0.5982	0.2007	0.5192	0.3651	0.2998

Table 6: Robustness parameter of SS, BH and ET studied for HPLC method.

SST Parameters	ET	SS	BH
Capacity factor (k)	8.51	11.66	51.8
Asymmetry (A_s)	1	1.7	1.5
Tailing factor (T_f)	1	1.36	1.25
HETP (mm)	0.0215	0.0066	0.0034
Separation factor (α)	8.93	-	45.19
Resolution (R_s)	3.78	-	34.19
Plate Count	3258.13	2564.41	2412.19

Table 7: System suitability parameters of SS, BH and ET for RP-HPLC method.

Formulation	Method	% Labeled claim obtained for SS	% Labeled claim obtained for BH	% Labeled claim obtained for ET
Eto-Salbetol	HPTLC	99.4091 ± 1.0956	98.3579 ± 0.3252	98.8969 ± 0.0437
	HPLC	99.93 ± 1.33	99.48 ± 0.57	97.34 ± 0.02

Table 8: Percent purity of SS, BH and ET in tablet dosage forms by HPTLC method.

Parameters	SS	BH	ET
Maximum	99.930	99.960	99.990
Minimum	98.750	99.600	99.550
Median	99.555	99.675	99.840
Mean	99.473	99.745	99.825
S.D.	0.405	0.159	0.166
S.E.	0.165	0.065	0.068
CI of mean	0.425	0.167	0.174
Skewness	-1.217	0.779	-0.836
Kurtosis	2.108	-1.854	0.269
K-S distribution	0.242	0.254	0.160
K-S probability	0.310	0.254	0.723
SWilk W	0.904	0.816	0.920
SWilk Probability	0.398	0.082	0.507

Table 9: Discriptive statistical analysis parameters of SS, BH and ET on HPTLC accuracy results

Parameters	SS	BH	ET
Maximum	99.790	99.900	99.930
Minimum	99.170	99.740	99.690
Median	99.440	99.855	99.725
Mean	99.443	99.847	99.767
S.D.	0.212	0.061	0.092
S.E.	0.087	0.025	0.038
CI of mean	0.222	0.064	0.097
Skewness	0.603	-1.154	1.405
Kurtosis	0.839	1.082	1.251
K-S distribution	0.210	0.226	0.280
K-S probability	0.487	0.396	0.148
SWilk W	0.965	0.860	0.830
SWilk Probability	0.860	0.190	0.107

Table 10: Descriptive statistical analysis parameters of SS, BH and ET on RP-HPLC accuracy results.

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