INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease that interferes with normal breathing and is not reversible. The chronic limitation of airflow is an important characteristic of COPD, which can be caused by a mixture of obstructive broncholiths (small airway disease) and emphysema (parenchymal destruction). COPD has extra-pulmonary effects, as it disrupts the structure of mucopolysaccharide fibers in mucoid sputum and produces less viscous mucus, which is easier to expectorate [2].

Salbutamol sulfate (SAL) is a beta-adrenoceptor agonist. Activation of β2-adrenergic receptors present in airway smooth muscle; results in the activation of adenylyl cyclase and lead to an increase in the intracellular concentration of cyclic-3',5'-adenosine monophosphate (cyclic AMP). There is an increase in cyclic AMP which results in the activation of protein kinase-A, which inhibits the phosphorylation of myosin and lowers intracellular ionic calcium concentrations, resulting in the relaxation. It is used for the relief of bronchospasm in a condition such as asthma and chronic obstructive pulmonary disease. Chemical name of SAL is 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl) phenol; sulfuric acid. It has a molecular formula of C_{10}H_{15}N_{2}O_{4}S and molecular weight of 576.702 g/mol.

Etofylline (ETO) is a xanthine bronchodilator which inhibits phosphodiesterase enzyme and intracellularly degrades cyclic nucleotides resulting in the intracellular accumulation of the cyclic AMP and causing bronchodilatation. Chemical name of ETO is 7-(2-hydroxyethyl)-1,3-dimethylpurine-2,6-dione. It has a molecular formula of C_{9}H_{9}N_{2}O_{4} and molecular weight of 224.22 g/mol.

Bromhexine hydrochloride (BROM) acts as an oral mucolytic agent. It disrupts the structure of mucopolysaccharide fibers in mucoid sputum and produces less viscous mucus, which is easier to expectorate [2]. Chemical name of BROM is 2,4-dibromo-6-[[cyclohexyl(methyl) amino]methyl]aniline, hydrochloride. It has a molecular formula of C_{13}H_{15}BrClN_{2}O_{4} and molecular weight of 412.594 g/mol.

SAL, ETO, and BROM are available in the market as a combined tablet dosage form, which is widely used in the treatment of asthma and COPD.

The literature survey reveals that high-performance liquid chromatography (HPLC) and ultraviolet (UV) methods were reported for the estimation of SAL, ETO, and BROM alone or in combination with other drugs in bulk and dosage forms.[4-14] However, so far there is no stability indicating method reported for the same. This initiated an interest to develop a new, simple, and rapid HPLC method of these drugs in combination with the marketed formulation used for the treatment of COPD and asthma. The proposed method was used successfully to separate the degraded products from the samples and it is optimized and validated as per the International Conference on Harmonization (ICH) guidelines [3] (Figs. 1-3).
EXPERIMENTAL CONDITIONS

Materials and reagents
SAL, ETO, and BROM were obtained as gift samples from Centaur Pharmaceuticals, Mumbai. A commercial preparation (ALBUTAMOL PLUS TABLET) used for analysis was procured from Pharmaceuticals market. Each tablet contains 2 mg of SAL, 200 mg of ETO, and 8 mg of BROM. HPLC grade acetonitrile (Thomas Baker) and water, potassium dihydrogen phosphate (LOBA CHEM), and orthophosphoric acid were used.

Instrumentation
The RP-HPLC chromatographic separation was carried out by Shimadzu prominence-i LC-2030 HPLC system containing software of LAB solution with pump p-5000, UV/VIS detector and a fixed injector equipped with 20 µL loop. The Lab Solution software was used for signal monitoring and processing.

Chromatographic conditions
- Column: Shim-pack C18 (250 x 4.6 mm, 5 µm)
- Mobile phase/acetonitrile: 0.1 M potassium dihydrogen phosphate buffer (35:65) adjusted to pH 3.0 with orthophosphoric acid
- Flow rate: 1.0 ml/min
- Wavelength: 225 nm
- Injection volume: 20 µL
- Runtime: 13 min
- Elution: Isocratic.

Preparation of 0.1 M potassium dihydrogen orthophosphate (pH 3.0)
About 13.609 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 1000 ml of water and adjusted pH with orthophosphoric acid to 3.0±0.05. The solution was then filtered using 0.45 µm membrane filter.

Preparation of mobile phase
The pH of (0.1 M) potassium dihydrogen orthophosphate was adjusted to 3.0 with orthophosphoric acid and mixed with acetonitrile in the proportion 65:35 and was sonicated.

Preparation of standard solution
100 mg of SAL, 100 mg of ETO, and 100 mg of BROM standard were accurately weighed and transferred into individual 100 ml volumetric flasks. About 70 ml of the mobile phase was added, sonicated to dissolve and diluted to 100 ml using mobile phase. Suitable dilutions were made to obtain a final concentration of 2,200 and 8 µg/ml SAL, ETO, and BROM, respectively.

Preparation of sample solution
10 tablets were weighed and powdered. The quantity of powder equivalent to 2 mg of SAL, 200 mg of ETO, and 8 mg of Bromhexine were transferred into a 100ml volumetric flask. About 70 ml of mobile phase was added, and the solution was sonicated for 30 min with intermittent shaking. The volume was made up using the mobile phase, mixed and filtered through 0.45 µ PVDF filter. Suitable dilutions were made to obtain a final concentration of 2200 and 8 µg/ml SAL, ETO, and bromhexine hydrochloride, respectively.

Statistical analysis
To evaluate the contribution of each factor with different levels on responses, two-way analysis of variance was performed using GraphPad Prism 7.04 software.

RESULTS AND DISCUSSION
The proposed RP-HPLC method was validated as per the ICH guidelines.

Selectivity and specificity
To assess the selectivity of the developed method solutions of all three drugs were injected into the system, three sharp peaks of SAL, ETO, and BROM were obtained at a retention time of 2.319, 2.698, and 10.329 min, respectively, in reference to the standard solution. Specificity was determined by comparison of the chromatogram of mixed standards and sample solutions Fig 4. As the retention time of standard drugs and the retention time of the drugs in sample solutions were same, so the method was specific. The parameters such as resolution (Rs) and asymmetric factor were calculated. A good correlation was found between the results of mixed standards and sample solutions. Results are shown in Table 1.

Linearity
The linearity of an analytical method has ability to obtain results, which are directly proportional to the concentration of an analyte in the sample. It was done by preparing the sample solutions containing 2 µg/ml, 200 µg/ml, and 8 µg/ml of SAL, ETO, and BROM, respectively. A calibration curve was drawn by plotting concentration on an X-axis versus area on Y-axis and regression equation, correlation coefficient, y-intercept, and slope of the equation were calculated. The result is shown in Table 2 and Figs. 6-8.

Accuracy
The accuracy of the proposed methods was estimated by recovery studies at three different levels, i.e. 80%, 100%, and 120%. The recovery studies were carried out by adding known amounts of standard SAL, ETO, and BROM and were added to the pre-analyzed samples, and they were subjected to a proposed HPLC method. The recoveries results of standards in pharmaceutical preparation are shown in Table 3.

Precision
The precision study was carried out to find out intraday and interday variations. The intraday and interday precision study of SAL, ETO, and BROM was carried out by estimating the correspondence response

Table 1: System suitability parameters

| Parameters         | SAL  | ETO  | BROM |
|--------------------|------|------|------|
| Retention time (min)| 2.319| 2.698| 10.329|
| USP plate count    | 3539 | 14621| 4683 |
| USP tailing        | 1.602| 1.219| 1.109|

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride

Table 2: Linearity studies

| Parameters         | SAL     | ETO     | BROM     |
|--------------------|---------|---------|----------|
| Linearity range (µg/ml) | 1.6–3.2 | 160–320 | 6.4–12.8 |
| Slope              | 542.63  | 35.048  | 334.00   |
| Intercept          | 288.46  | 22.877  | 2.309    |
| Correlation coefficient | 0.998  | 0.998   | 0.999    |

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride

---

**Graph Pad**

Fig. 1: Structure of salbutamol

**Table 3**

| Parameters         | SAL  | ETO  | BROM |
|--------------------|------|------|------|
| System suitable parameters |     |      |      |
| Retention time (min) | 2.319| 2.698| 10.329|
| USP plate count    | 3539 | 14621| 4683 |
| USP tailing        | 1.602| 1.219| 1.109|

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride

**Table 4**

| Parameters         | SAL     | ETO     | BROM     |
|--------------------|---------|---------|----------|
| Linearity range (µg/ml) | 1.6–3.2 | 160–320 | 6.4–12.8 |
| Slope              | 542.63  | 35.048  | 334.00   |
| Intercept          | 288.46  | 22.877  | 2.309    |
| Correlation coefficient | 0.998  | 0.998   | 0.999    |

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride
3 times on the same day and on 3 different days for 3 different concentrations, and the results were reported in terms of percentage relative standard deviation (% RSD), however, all results fall within an acceptance limit (RSD <2), as shown in Table 4.

Limit of detection (LOD) and limit of quantification (LOQ)
LOD is the ability of analytical method able to detect the lowest concentration of the analyte. LOQ is the lowest concentration of the analyte which can be quantitatively analyzed with acceptable precision and accuracy. It was calculated based on the slope and blank response.
from the calibration curve as per the ICH guidelines. LOD and LOQ were calculated based on the standard deviation of the response and slope. The result is shown in Table 4.

**Robustness**

The robustness study was done by making small changes in the optimized method parameters like ±0.2 ml change in flow rate, ±2°C

| Parameters       | Precision (% RSD) | SAL  | ETO  | BROM |
|------------------|-------------------|------|------|------|
| Intraday (n=3)   | 0.85              | 0.41 | 0.50 |
| Interday (n=3)   | 0.63              | 0.82 | 0.54 |
| LOD              | 0.10              | 8.10 | 0.30 |
| LOQ              | 0.31              | 24.56| 0.91 |

LOD: Limit of detection, LOQ: Limit of quantification, SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride, % RSD: % Relative standard deviation

| Table 3: Results of accuracy studies |
|-------------------------------------|
| Pre-analyzed sample solution (µg/ml) | Sample concentration (µg/ml) | Excess drug added (µg/ml) | Amount recovered (µg/ml) | % recovery |
|-------------------------------------|-----------------------------|--------------------------|--------------------------|------------|
| SAL                                 | 1                           | 0.8                      | 1.8                      | 100.39     |
| ETO                                 | 100                         | 100                      | 200                      | 98.25      |
| BROM                                | 4                           | 3.2                      | 7.2                      | 99.90      |

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride

**Table 4: Results of precision and LOD and LOQ**

| Parameters       | Precision (% RSD) | SAL  | ETO  | BROM |
|------------------|-------------------|------|------|------|
| Intraday (n=3)   | 0.85              | 0.41 | 0.50 |
| Interday (n=3)   | 0.63              | 0.82 | 0.54 |
| LOD              | 0.10              | 8.10 | 0.30 |
| LOQ              | 0.31              | 24.56| 0.91 |

LOD: Limit of detection, LOQ: Limit of quantification, SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride, % RSD: % Relative standard deviation

**Table 5: Assay determination of SAL, ETO, and BROM**

| Brand% amount found | Sample concentration (µg/ml) | Excess drug added (µg/ml) | Amount recovered (µg/ml) | % recovery |
|--------------------|------------------------------|---------------------------|--------------------------|------------|
| Albutamol plus     | 1                            | 0.8                       | 1.8                      | 100.39     |
| (2 mg SAL+200 mg ETO+8 mg BROM) | 100              | 100                      | 200                      | 98.25      |

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride

**Fig. 10: Chromatograph of base degradation**
change in temperature, and ±2 nm change in wavelength. There was no significant impact on the retention time and tailing factor.

**Assay**

The amount of SAL, ETO, and BROM per tablet was calculated by comparing the peak area of the standard solution and sample. The result is shown in Table 5.

**Forced degradation studies**

Forced degradation studies were carried out by treating the sample under the following conditions: Sample was subjected to acid degradation using 1 N HCl, base degradation using 1 N NaOH, oxidative degradation using 3.0% v/v of H₂O₂, photolytic degradation by exposing the sample in UV light for 1 day, thermal degradation by heating at 105°C for 1 h, humidity degradation using 25°C, and 80% RH in a humidity chamber. The results of stress studies were shown in Table 6 and Figs. 9-14.

**CONCLUSION**

In the present study, stability indicating RP-HPLC method has been developed and validated for simultaneous estimation of SAL, ETO, and BROM in the pharmaceutical dosage form. The developed method was validated as per the ICH guidelines, and the results were within limits. The stress testing studies revealed that the method was successfully employed to resolve the degraded products from the sample. This method can be utilized in routine quantitative and qualitative analysis of SAL, ETO, and BROM simultaneously and method can reduce the time for routine quality control analysis in their dosage form.

**ACKNOWLEDGMENT**

The authors are thankful to Centaur Pharmaceuticals for kindly providing gift samples of SAL, ETO, and BROM.

**AUTHOR’S CONTRIBUTION**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Kajal D. Gawdecollected and analyzed the data, performed laboratory work and wrote introduction, discussion, material, and method part.

**CONFLICTS OF INTEREST**

The authors declare that there are no conflicts of interests regarding the publication of this paper.

**REFERENCES**

1. Vijayan VK. Chronic obstructive pulmonary disease. Indian J Med Res 2013;137:251-69.
2. Tripathi KD. Essentials of Medical Pharmacology. New Delhi: JP Medical Ltd.; 2013.
3. International Conference on Harmonization, Harmonized Tripartite Guideline, Validation of Analytical Procedures, Text and Methodology, Q2 (R1), November; 2005.
4. Maithani M, Singh R. Development and validation of a stability-indicating HPLC method for the simultaneous determination of salbutamol sulphate and theophylline in pharmaceutical dosage forms. J Anal Bioanal Techniques 2011;3:11.
5. Venkatesh V, Prabahar E, Suresh PV, Umamaheshwari CH, Rama NR. A new RP-HPLC method for simultaneous estimation of etofylline and theophylline in tablets. Res J Pharm Tech 2011;4:28-30.
6. Chitlange S, Pandikar S, Tawar M, Wankhede S. Simultaneous spectrophotometric estimation of salbutamol sulphate and ambroxol hydrochloride by using various solvent in bulk as well as in tablet formulation. Asian J Res Chem 2011;4:746-9.
7. Susmitha K, Thirumalachary M, Venkateshwarlu G. Spectrophotometric determination of bromhexine HCl in pure and pharmaceutical forms. ISRN Anal Chem 2013;2013:7.
8. Chitlange SS, Chaturvedi KK, Tawargeri SR, Wankhede S. UV spectroscopic and Stability-indicating TLC-densitometric method for simultaneous estimation of salbutamol sulphate and prednisolone in pharmaceutical dosage form. Asian J Res Chem 2011;4:786-90.
9. Prusty A, Chennupati SV, Sathpathy J. UV-Visible spectrophotometric method development and validation of assay for Etofylline tablet formulation. J Appl Chem 2014;3:2020-8.
10. Al-Ward HS. Spectrophotometric method for the determination of bromhexine hydrochloride in pure and pharmaceutical preparations. Iraqi J Sci 2011;52:400-7.
11. Jain V, Sharma MC. Validated RP-HPLC method for determining the levels of bromhexine HCl, chlorpheniramine maleate, dextromethorphan HBr and guaiphenesin in their pharmaceutical dosage forms. J Taibah Univ Sci 2016;10:38-45.
12. Gandhi SV, Mittal PS, Gaikwad AM. Development and validation of stability indicating rp-hplc method for simultaneous estimation of beclomethasone dipropionate and salbutamol sulphate. Int J Pharm Pharm Sci 2015;7:252-7.
13. Deosarkar AV, Deshpande SD, Walode SG, Tuljapure DS, Tekale SG, Waghmode VM. Simultaneous quantification of salbutamol sulphate and ambroxol hydrochloride by rp-hpc and hptlc in bulk drug and dosage form. Int J Pharm Pharm Sci 2012;4:217-21.
14. Chitlange SS, Chaturvedi KK, Wankhede SB. Development and validation of spectrophotometric and HPLC method for the simultaneous estimation of salbutamol sulphate and prednisolone in tablet dosage form. J Anal Bioanal Tech 2011;2:2.

---

**Table 6: Force degradation of SAL, ETO, and BROM**

| S.NO | Stress condition       | SAL | ETO | Bromhexine hydrochloride |
|------|------------------------|-----|-----|--------------------------|
|      |                        | % assay | % difference w.r.t assay | % assay | % difference w.r.t assay | % assay | % difference w.r.t assay |
| 1    | Control                | 99.64 | NA  | 99.57                    | NA      | 99.79                    | NA      |
| 2    | Acid degradation       | 94.85 | 4.78 | 91.43                    | 8.13    | 87.11                    | 12.67   |
| 3    | Base degradation       | 98.51 | 1.12 | 88.77                    | 10.79   | 84.23                    | 15.55   |
| 4    | Oxidative degradation  | 96.72 | 2.91 | 91.67                    | 7.89    | 87.96                    | 11.82   |
| 5    | Photolytic degradation | 93.53 | 6.10 | 98.51                    | 1.05    | 95.13                    | 4.65    |
| 6    | Thermal degradation    | 95.32 | 4.31 | 92.63                    | 87.98   | 6.93                     | 11.00   |
| 7    | Humidity degradation   | 96.86 | 2.77 | 94.40                    | 5.16    | 89.18                    | 10.60   |

SAL: Salbutamol sulfate, ETO: Etofylline, BROM: Bromhexine hydrochloride