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Analytical Methods Development and Validation For Simultaneous Quantification of Budesonide and N-Acetylcysteine In Combination

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ABSTRACT

Clinical studies had demonstrated that the combination of Budesonide and N-Acetylcysteine offers improved therapeutic efficacy in children with refractory Mycoplasma pneumoniae pneumonia, with a reduced incidence of adverse effects compared to monotherapy. However, no any validated analytical method is currently available for the simultaneous qualitative and quantitative estimation of these drugs in combination. Therefore, the aim of this study was to develop and validate analytical methods for the simultaneous estimation of Budesonide and N-Acetylcysteine in combination. An innovative, straightforward, accurate, precise, sensitive, and robust analytical approaches including first order derivative UV spectrophotometry and reversed-phase HPLC (RP-HPLC) were explored. The first order derivative UV spectrophotometric method involved the determination of both drugs at their respective zero crossing points (ZCP). The first order derivative spectrum was obtained in methanol, and the determinations were made at the ZCP of Budesonide at 210 nm for the measurement of N-Acetylcysteine and at the ZCP of N-Acetylcysteine at 267 nm for the measurement of Budesonide. Linearity was obeyed in the concentration range of 0.24-1.2 µg/mL for Budesonide at 267 nm and 20-100 µg/mL for N-Acetylcysteine at 210 nm. An Isocratic RP-HPLC approach was employed using reversed-phase Kromstar C18 (250 × 4.6 mm, 5 µm) column as a stationary phase and detection wavelength at 216 nm for the two analytes in the chromatographic column. The mobile phase comprised of (60:20:20 %v/v/v) mixture of Methanol: Acetonitrile: Phosphate buffer (pH: 3.5 adjusted with 10% ortho phosphoric acid) at 216 nm. The elution was carried out at a constant flow rate of 1 mL/min. Furthermore, the two advanced methods underwent statistical comparison by student's t-test and validated by the valuable ICH Q2 (R2) guideline, affirming their effectiveness as a successful analytical tool for the concurrent analysis of both in bulk and the synthetic mixture fabricated in the laboratory.

KEYWORDS: Budesonide (BUDE); N-Acetylcysteine (NAC); First Order Derivative UV spectrophotometry, Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

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INTRODUCTION

Chronic inflammatory airway diseases, including COPD, chronic bronchitis, and asthma, constitute a major global and national health burden, particularly in developing countries such as India due to smoking, biomass fuel exposure, and increasing air pollution. These diseases are characterized by persistent airway inflammation, oxidative stress, and excessive mucus production, leading to recurrent exacerbations and reduced quality of life [1]. Mycoplasma pneumoniae pneumonia (MPP) is a common cause of community-acquired pneumonia in children. Although most cases respond well to macrolide antibiotics, a subset of patients develop refractory Mycoplasma pneumoniae pneumonia (RMPP), characterized by persistent fever, worsening respiratory symptoms, and progressive lung inflammation despite appropriate antibiotic therapy. Excessive immune response, airway inflammation, and mucus hypersecretion play a crucial role in disease progression [2]. Budesonide, chemically 11 β, 21-dihydroxy-16α,17α-[butane-1,1-diylbis(oxy)] pregna-1,4-diene-3,20-dione (Figure 1A), is a glucocorticoid used for long-term management of asthma and COPD. It inhibits multiple inflammatory pathways by suppressing cytokines and inflammatory mediators. Its high first-pass hepatic metabolism reduces systemic bioavailability and adverse effects. Due to alkyl substitution at C-22, budesonide exists as 22R and 22S epimers [1, 3]. N-Acetylcysteine (NAC) is a synthetic derivative of the amino acid L-cysteine, widely used for the treatment of paracetamol overdose and as a mucolytic in chronic bronchopulmonary disorders such as bronchitis and pneumonia. It exhibits mucolytic, antioxidant, and antidotal properties and has been used therapeutically for several decades. Chemically, NAC is (2R)-2-acetamido-3-sulfanylpropanoic acid (Figure 1B) [1, 3].

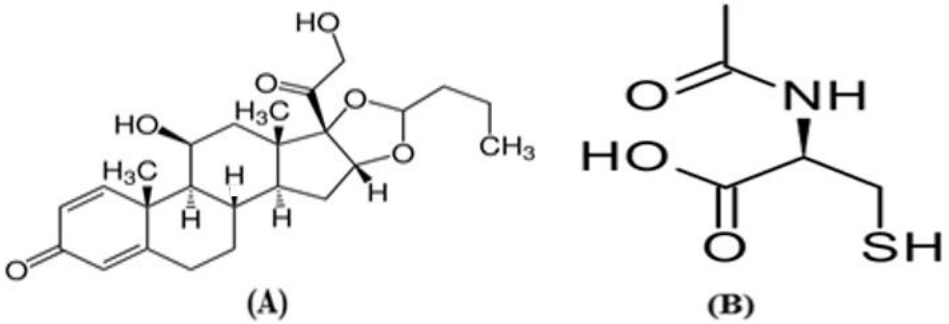

Figure 1: Chemical Structure: (A) Budesonide and (B) N-Acetylcysteine

Combination of Budesonide (BUDE) with N-Acetylcysteine (NAC) was studied under clinical trial phase and was proved that the combination is effective refractory Mycoplasma pneumoniae pneumonia in children [4]. Budesonide and N-acetylcysteine nebulizers are frequently used as lavage agents during bronchoscopic alveolar lavage. BLA-coupled BUDE & NAC was superior to the other two groups in enhancing the effectiveness of RMPP in children, which might increase lung opacity absorption and minimize lung inflammation [5]. Combination of BUDE and NAC improved LPS-induced ALI by inhibiting pulmonary neutrophil recruitment better than treatment alone. Budesonide (BUDE) and N-acetylcysteine (NAC) are often used in the treatment of bronchopneumonia [6-7]. The combination of Budesonide and N-acetylcysteine is clinically important in these populations as it simultaneously targets airway inflammation and mucus hypersecretion, two critical factors driving disease progression and morbidity. Hence, this combination holds significant therapeutic relevance in high-risk and vulnerable populations.

Budesonide is official in the United States Pharmacopoeia (USP) [8] and Indian Pharmacopoeia (IP) [9], both of which describe an HPLC assay method for its estimation [8-9]. Several analytical methods had been reported in the literature, including selective UV spectrophotometric methods for pure Budesonide [10], High-performance liquid chromatographic methods [11], RP-HPLC method for simultaneous estimation of Budesonide with Formoterol [12], and HPTLC method for its simultaneous estimation with Levalbuterol hydrochloride in combined dosage form [13].

N-Acetylcysteine is official in the USP with LC assay method [8]. Reported analytical methods included LC-UV estimation without derivatization [14], absorption correction UV method for simultaneous estimation with Ambroxol hydrochloride [15], Q-absorbance ratio UV method with Acebrophylline [16], RP-HPLC method with Cefixime [17] and L-arginine [18], and an HPTLC method for simultaneous determination with Taurine [19].

From extensive literature survey, there is no any analytical method available in this combination. Given the widespread use of Budesonide and N-acetylcysteine in the management of chronic respiratory disorders, the development of validated analytical methods for their simultaneous estimation is essential to ensure quality, safety, and efficacy of this combination intended for large patient populations. The objectives of the present work were to develop and validate a linear, accurate, precise, and sensitive first-order derivative UV method and RP-HPLC method for the simultaneous estimation of Budesonide and N-Acetylcysteine in synthetic mixture, providing a practical and robust solution for routine laboratory applications. These methods were validated according to ICH Q2 (R2) guideline [20] within all Validation Parameters [21-23]. All methods were compared statistically using student's t-test [24] to determine their efficacy and practicality, with a focus on quality control research.

EXPERIMENTAL MATERIALS

All chemicals and reagents used in the present study were HPLC grade and analytical grade to ensure accuracy and reproducibility of the results. Acetonitrile, methanol, and water (HPLC grade) were procured from Finar Chemicals Pvt. Ltd., India, used for the preparation of mobile phases, diluents, and standard solutions. Ortho phosphoric acid of analytical reagent (AR) grade, obtained from Astron Chemical India, was employed for pH adjustment of the phosphate buffer. Budesonide, used as the reference standard, was kindly supplied by Cadila Pharmaceuticals Ltd., Dholka, while N-acetylcysteine was procured from Ahem Lifecare LLP., Ahmedabad.

Instruments & Software

The spectrophotometric measurements were performed using a UV-Visible spectrophotometer (Shimadzu-1900, UV Probe 2.7 version software) with a spectral bandwidth of 1 nm was employed for all spectroscopic measurements, using a pair of 1.0 cm matched quartz cells over the range of 200-400 nm. For chromatographic information acquisition and analysis, High-Performance Liquid Chromatography system Systronic RP-HPLC (SYS-LC-138) with UV Detector was utilized together. The pH of the buffer solution was observed utilizing the Chemi Line pH meter. The Scale-Tec analytical balance was utilized to weigh the samples. The HPLC mobile phase was subjected to sonication using an Sonicator- Digital Pro+, PS-10A, (Broleo).

Analytical conditions

In accordance with ICH Q2 (R2) requirements [20], the analytical conditions for a simultaneous technique for the measurement of Budesonide and N-acetylcysteine in UV and HPLC were optimized and validated. For UV Spectroscopy Methanol was used

as a Solvent. Detection wavelength (λmax) of BUDE and NAC were 243 nm and 202 nm, respectively. The first-order derivative UV spectra were derived from the zero-order spectra using methanol as the solvent. Quantitative analysis was performed at the zero-crossing point (ZCP) of Budesonide at 210 nm for the estimation of N-acetylcysteine, and at the ZCP of N-acetylcysteine at 267 nm for the estimation of Budesonide. For RP-HPLC, Kromstar C18 (250 mm × 4.6 mm, 5 µm) was used in the procedure. The mobile phase consisted of Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v), 216 nm wavelength was selected for RP-HPLC, with 1 mL/min flow rate.

Preparation of Solutions:

Preparation of Stock Solution: Accurately weighed 10 mg of Budesonide (BUDE) and 10 mg of N-acetylcysteine (NAC) were individually transferred into separate 100 mL volumetric flasks and dissolved in methanol. The solutions were sonicated to ensure complete dissolution, and the volume was made up to the mark with methanol to obtain standard stock solutions having a concentration of 100 µg/mL of BUDE and 100 µg/mL of NAC, respectively.

Preparation standard solution: Pipetted out 0.048 ml solution of Budesonide (100 µg/mL) and 4 ml standard stock solution of N-Acetylcysteine (100 µg/mL) into different 10 ml volumetric flask and diluted up to mark with Methanol to get the 0.48 µg/mL of Budesonide and 40 µg/mL of N-Acetylcysteine.

Preparation of standard working solution: To produce concentration ranges of 0.24-1.2 µg/mL of BUDE and 20-100 µg/mL of NAC, From each stock solution, BUDE (0.024, 0.048, 0.072, 0.096 and 0.12 ml) and NAC (2, 4, 6, 8 and 10 ml) were pipetted out in ten different 10 ml volumetric flasks and made up to mark with Methanol to obtained 0.24, 0.48, 0.72, 0.96 and 1.2 µg/mL of BUDE and 20, 40, 60, 80 and 100 µg/mL for NAC, respectively. Under the optimized spectrophotometric conditions, the samples were analyzed using a 1 cm quartz cuvette in the UV spectrophotometer. Similarly, the optimized chromatographic conditions, 20 µL of each standard working solution was injected into the RP-HPLC system.

METHODOLOGY

Method I: UV-spectrophotometric method: First Order Derivative Method was selected for simultaneous estimation of Budesonide and N-Acetylcysteine in Synthetic Mixture. Each working standard solution was scanned individually over the wavelength range of 200-400 nm. In zero order UV spectra, Budesonide exhibited an absorption maximum at 243 nm (Figure 2), close to the reported value of 246 nm, while N-acetylcysteine showed an absorption maximum at 202 nm (Figure 3). BUDE and NAC standard stock solutions were prepared in Methanol at concentrations of 100 µg/mL and 100 µg/mL, respectively. A small amount of each stock solution was taken and placed into 10 mL volumetric flasks. Methanol was used to adjust the volumes to the mark, resulting in final concentrations of BUDE ranging from 0.24 to 1.20 µg/mL and NAC ranging from 20 to 100 µg/mL. All zero-order absorption UV spectra were converted to first-order derivative UV spectra. Calibration functions were established by plotting first-order derivative absorbance against corresponding concentrations for each analyte. Appropriate volume, 0.048 mL of Budesonide and 4 ml N-Acetylcysteine standard stock solution was transferred to two separate 10 mL volumetric flasks and the volume was adjusted to mark with methanol to get concentration 0.48 and 40 µg/mL, respectively. The solutions were scanned separately in the UV-region i.e., 400-200 nm. The zero-order UV absorption spectra of BUDE and NAC in Methanol shown in Figure 4. The zero-order spectrum was processed to obtain first-derivative spectrum. The two first derivative spectra were overlaid which shows that Budesonide showed zero crossing at 210 nm, while N-Acetylcysteine showed zero crossing at 267 nm which shaws in Figure 5. The determinations were made at 267 nm for Budesonide (ZCP of N-Acetylcysteine) and 210 nm for N-Acetylcysteine (ZCP of Budesonide). The first order overlay UV spectra of Budesonide and N-Acetylcysteine are presented in Figure 8 and 9, respectively.

Method II: Reverse Phase High Performance Liquid Chromatography Method: For RP-HPLC, the analysis was carried out using an isocratic elution technique using a mobile phase comprised of different mobile phases such as Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) at a flow rate of 1 mL/min found better separation of both the drug peaks. Prior to usage, the solvents were filtered through a 0.45 m filter and sonicated for 30 min. The stationary phase was a Kromstar C18 (250 mm × 4.6 mm, 5 µm), and the eluent was observed by a U.V Detector from 200 to 400 nm, alongside chromatograms extracted at 216 nm. The calibration curves were prepared by measuring the peak areas of BUDE and NAC and plotted their values against the pertinent concentrations. In accordance, the equations for linear regression were calculated.

Method Validation: The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), ICH Q2(R2): Validation of Analytical Procedures [20] established standards for the validation of the analytical procedures utilized in this investigation.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

Linearity and Range: (n=6): The linearity of Budesonide and N-Acetylcysteine was found to be in the range of 0.24-1.2 µg/mL and 20-100 µg/mL, respectively. Plot the calibration curve of peak area vs. concentration (µg/mL). Linearity of both the drugs were checked in term of slope, intercept and correlation coefficient.

Precision: The Intraday and Interday precisions also referred to as repeatability and intermediate accuracy, respectively were used to assess the precision of Methods I and II. The experiment was conducted on the same day and for the next three days for

both Intraday and Interday precision, analysing freshly made solutions at concentrations of 0.24, 0.48, and 0.72 µg/mL of BUDE and 20, 40, and 60 µg/mL of NAC. To assess intermediate precision, the mean absorbance (UV) and peak area (HPLC) were recorded for each set of experiments. For repeatability, 0.48 µg/mL of BUDE and 40 µg/mL of NAC were used. The results were represented as a percentage Relative Standard Deviation (RSD), with a value of less than two considered acceptable. This meticulous approach ensures a comprehensive evaluation of the precision of the analytical methods, providing confidence in the reliability and consistency of the results obtained for the concentrations of BUDE & NAC in the tested solutions.

Limit of Detection (LOD): Limit of detection can be calculated using following equation as per ICH guidelines.

$$LOD = 3.3*\sigma/S$$

Where, σ = standard deviation of the calibration curve
S = slope of the calibration curve

Limit of Quantification (LOQ): Limit of quantification can be calculated using following equation using the standard deviation of the Y-intercept (σ) and the mean slope (S) of the calibration curve according to ICH Q2 (R2) guideline.

$$LOQ = 10*\sigma/S$$

Accuracy (Recovery study) (n=3): The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50 %, 100 %, 150 % and the values were measured for Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL). This performance was done in triplicate. The accuracy of the method was determined by calculating recovery of Budesonide and N-Acetylcysteine by the standard addition method.

Assay as analysis of Synthetic Mixture: The synthetic mixture of Budesonide and N-Acetylcysteine was prepared in the ratio of 0.12:10. A synthetic mixture equivalent to 100 mg was prepared by accurately weighing Budesonide (0.12 mg) and N-Acetylcysteine (10 mg). Microcrystalline Cellulose (16.03 mg), Lactose (11.20 mg), Magnesium Stearate (9.95 mg), Talc (6.22 mg), and Croscarmellose Sodium (6.22 mg) were used as excipients. All the components were transferred into a mortar and blended thoroughly using a pestle to obtain a homogeneous synthetic mixture. This mixture was transferred in 100 ml volumetric flask and allowed to sonicate and made up to mark with Methanol. This solution was filtered through Whatmann filter paper. The filtrate was diluted to the mark with Methanol. The mixture contains 1.2 µg/mL of Budesonide and 100 µg/mL of N-Acetylcysteine.

Preparation of sample solution: Accurately 4 ml from the above solution [mixture of Budesonide (1.2 µg/mL) and N-Acetylcysteine (100 µg/mL)] was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Water. Final concentration of Budesonide was 0.48 µg/mL and N-Acetylcysteine 40 µg/mL then analyzed using the previously described UV-spectrophotometric and chromatographic conditions. The concentrations of BUDE and NAC were calculated using a regression equation.

Robustness: The robustness of analytical methods becomes evaluated to decide their ability to face up to minor variations in approach situations. For the HPLC technique, samples have been subjected to evaluation below changed situations, which include adjustments inside the flow rate (± 0.1 mL/min), detection wavelength (± 2 nm), and natural content material (± 2 %) inside the mobile segment. The resulting results on machine suitability parameters have been intently monitored. In the times of Methods I and II, distinct analysts conducted sample analyses to evaluate the robustness of the strategies.

System Suitability Tests: A system suitability test is an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The test includes the Resolution, Column efficiency, Tailing factor and Theoretical plates.

Statistical Comparison of Methods: Statistical analysis was performed to identify significant differences among the developed analytical methods. A statistical analysis was conducted using the student's t-test [24] to compare the results of accuracy and assay for proposed UV spectrophotometric and HPLC methods. A statistical test (Student's t-test) [24] was applied to evaluate the significance of difference between the two methods. The calculated t-value was compared with the theoretical t-value at a 95% confidence level. The student's t-test was calculated using the following formula:

$$T = \frac{|\bar{X}_1 - \bar{X}_2|}{S_P \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where, 〖X〗_1 and 〖X〗_2 were Mean value obtained from UV and RP-HPLC methods as two groups, n1 and n2 were number of observations for both methods, respectively and Sp was pooled standard deviation. The pooled standard deviation was calculated using the formula:

$$SP = \sqrt{\frac{(n_1 - 1)(S_1)^2 + (n_2 - 1)(S_2)^2}{n_1 + n_2 - 2}}$$

Where, S_1 and S_2 were standard deviation of the proposed methods as two groups.

RESULTS AND DISCUSSION

Method I: UV Method: In pharmaceutical analysis, the simultaneous estimation of multiple components using UV spectroscopy is a widely utilized method. Various techniques, including the Simultaneous Equation, Derivative Spectrophotometric approach and the absorbance ratio method, are employed for this purpose. The simultaneous estimation using UV visible spectroscopy offers several advantages, including ease of use, cost-effectiveness, and minimal time and labor requirements. These attributes make UV visible spectroscopic methods particularly valuable in pharmaceutical research and quality control, allowing for efficient and economical simultaneous determination of multiple components in a given sample.

Selection of wavelength for Budesonide and N-acetylcysteine: The remarkable absorbance of Budesonide exhibited an absorption maximum at 243 nm (Figure 2), while N-acetylcysteine showed an absorption maximum at 202 nm (Figure 3).

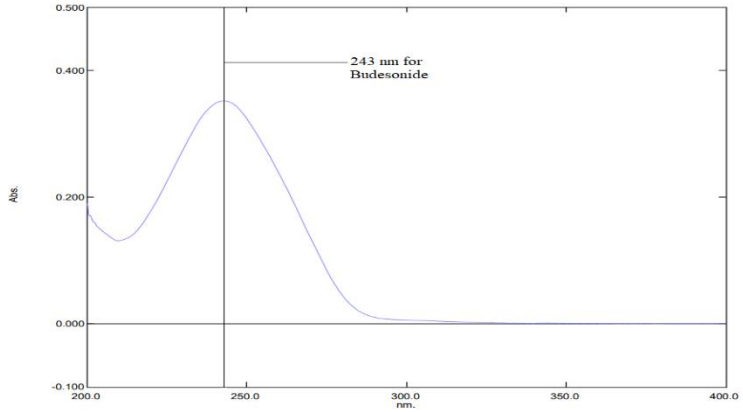

Figure 2: UV Spectrum of Budesonide (0.48 µg/mL) at 243 nm

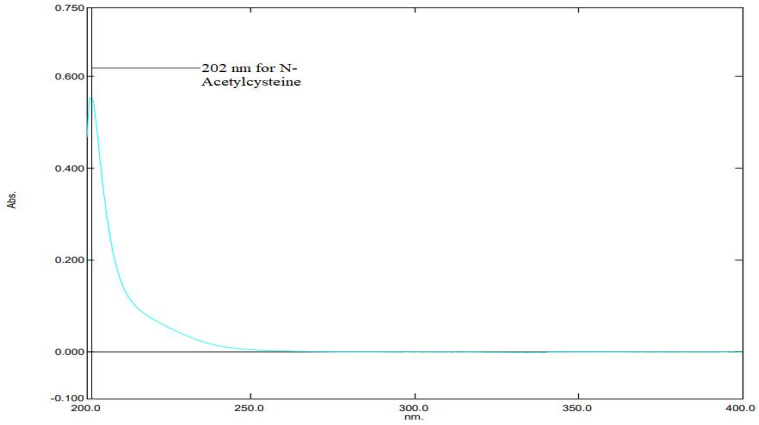

Figure: 3: UV Spectrum of N-Acetylcysteine (40 µg/mL) at 202 nm

The zero-order UV absorption spectra of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol was showed in Figure 4.

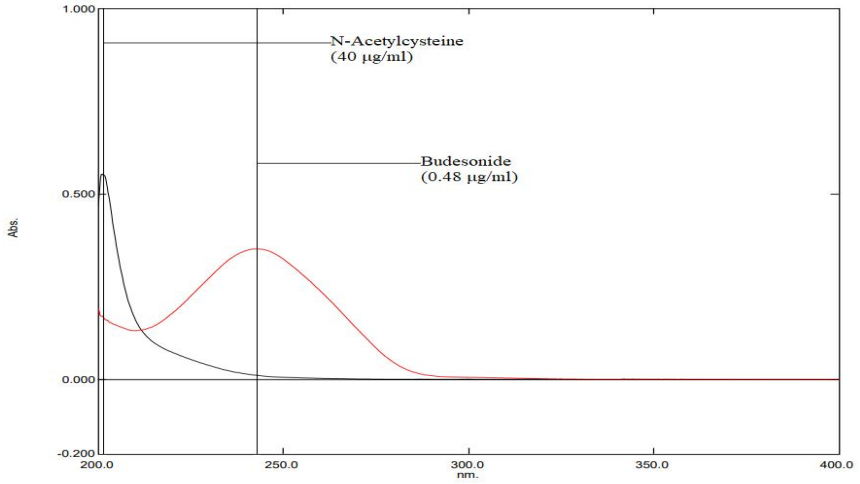

Figure 4: Overlain UV Spectra of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol (Zero Order)

First order derivative UV Method Development: The BUDE and NAC overlapping absorption throughout the 200 - 400 nm range is shown by these spectra, which makes it more difficult to quantify the pharmaceuticals using traditional UV spectrophotometry without accounting for the overlap. The sum of the absorbances of the two compounds may be used to calculate the overall absorbance of a solution containing a combination of both at a certain wavelength. In situations where the levels of the two medicinal drugs overlap, the method entails figuring out the quantity of each drug using their zero-order spectra. The resulting absorbance spectra were derived to eliminate the interference of absorbing species. The first derivative corresponding to each absorption spectrum of each drug was recorded, using $\Delta\lambda$ = 2 nm and scaling factor 4. The amplitude values were measured at 267 nm (λ1) (ZCP of NAC) for BUDE and 210 (λ2) (ZCP of BUDE) for NAC showed in Figure 5.

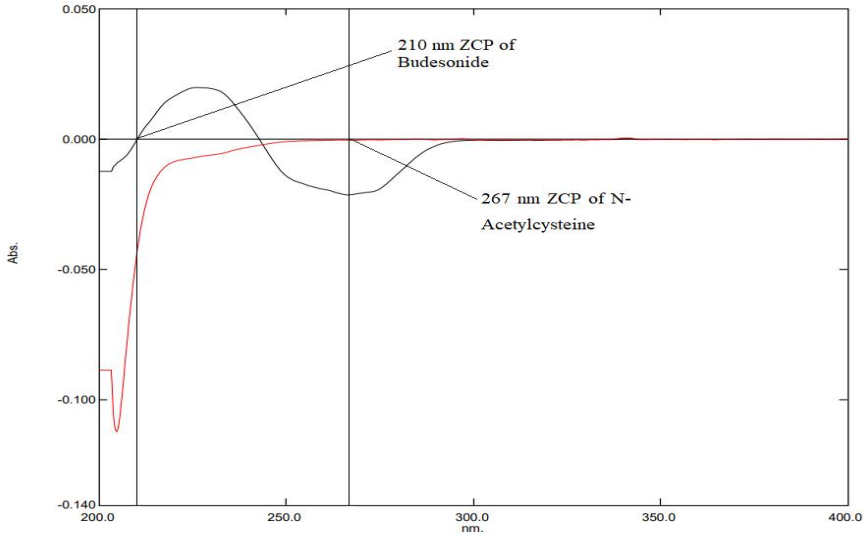

Figure 5: Overlain UV Spectra of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol (First Order)

Method II: RP-HPLC Method: Pharmaceutical analysis commonly uses simultaneous estimation using RP-HPLC. It enables the use of RP-HPLC to determine the presence of many chemicals in a sample. For the simultaneous estimate of various components, including medications and their contaminants, in pharmaceutical formulations, a number of techniques have been devised and proven effective. Utilizing an appropriate column, mobile phase, and detection equipment, the simultaneous estimation technique by HPLC allows for the separation and quantification of the target substances. In pharmaceutical analysis, Reverse Phase high-performance liquid chromatography (RP-HPLC) is a great instrument for simultaneous estimation that offers confidence and specificity for the identification of chemical entities in Synthetic Mixture.

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also performs better in terms of efficiency, stability and reproducibility. C18 column was selected because it is least polar compare to C4 and C8 columns.C18 column allows eluting polar compounds more quickly compare to non-polar compounds. In addition to this UV detector is used which allows easy detection of the compounds in UV transparent organic solvents. Hence, C18 (250×4.6 mm) column of 5µm particle packing was selected for separation of Budesonide and N-Acetylcysteine. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

Selection of detection wavelength: The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 216 nm both drugs give good peak height and shape. So, 216 nm was selected for simultaneous estimation of Budesonide and N-Acetylcysteine in synthetic mixture.

Overlain UV Spectra of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol has been shown in Figure 6.

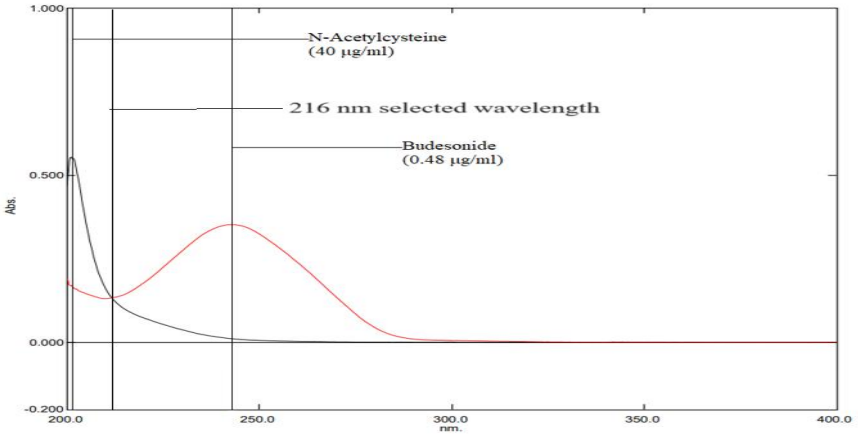

Figure 6: Selection of detection wavelength at 216 nm from Overlain UV Spectra of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol

RP-HPLC Method Development: Liquid chromatography coupled with UV detection was used to develop a way for simultaneously measuring BUDE and NAC. Achieving acceptable peak symmetry and theoretical plates within a realistic time period was the aim. The chromatographic conditions were optimized by experimenting with various stationary and mobile phases. The Kromstar C18 (250×4.6 mm, 5 µm) column had the best separation with symmetric peaks and the shortest retention period among the reversed phase C8 and C18 columns that were studied. A combination of Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) was the ideal mobile phase. Mixtures of Methanol: Acetonitrile: Phosphate buffer were also attempted; however, the outcome was asymmetric peaks and a prolonged retention period of BUDE & NAC. where NAC eluted first at 3 min, followed by BUDE at 6.6 min, showed in Figure 7. The RP-HPLC system formed satisfactory system suitability parameters such as [tailing factor was less than 2, Column efficiency was more than 2000, resolution was more than 2].

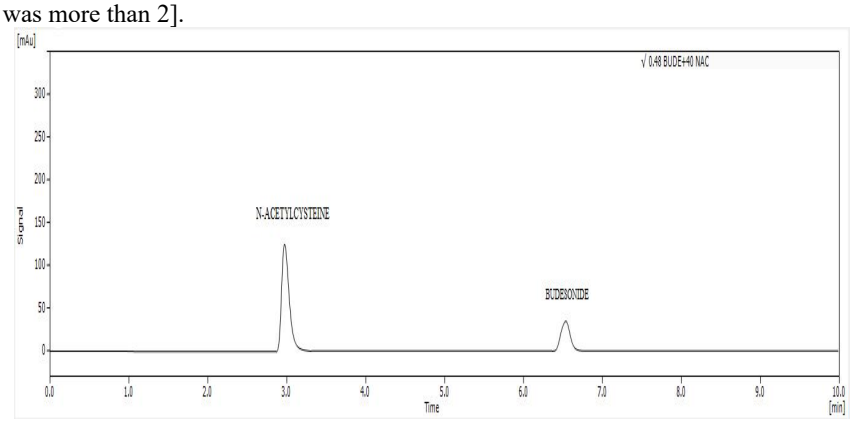

Figure 7: RP-HPLC Chromatogram of Budesonide (0.48 µg/mL) and N-Acetylcysteine (40 µg/mL) in Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) at 216 nm {Run time: 10 min, Flow rate: 1 ml/min}

VALIDATION OF THE PROPOSED METHODS

Validation Parameters of the UV Method:

Linearity and range: For BUDE and NAC, the absorbances ranged from 0.24 - 1.2 µg/mL at 267 nm and 20 to 100 µg/mL at 210 nm showed in Figure 8 and 9, respectively.

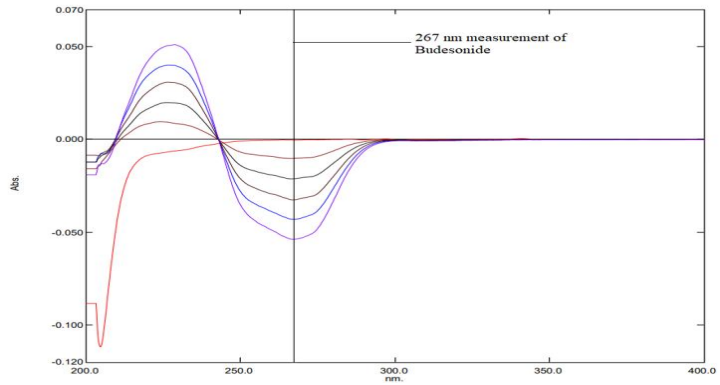

Figure 8: Overlain UV Spectra of Budesonide (0.24-1.2 µg/mL) at 267 nm

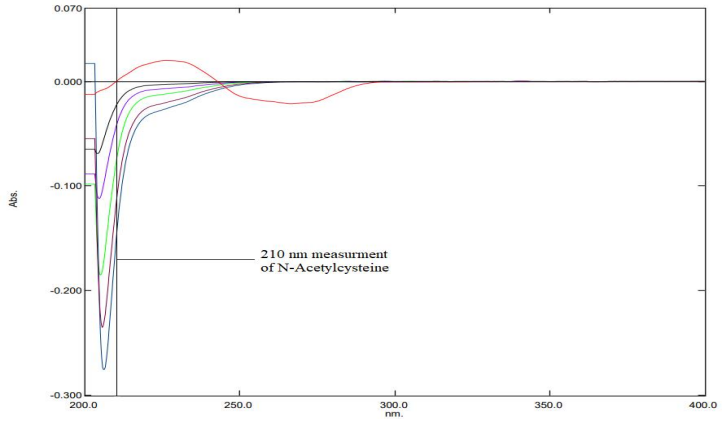

Figure 9: Overlain UV Spectra of N-Acetylcysteine (20-100 µg/mL) at 210 nm

A linear relationship was found and calibration curve was plotted for concentration vs. absorbance. For BUDE, the calibration curve equation y = 0.0458x + 0.0001, while for NAC, it was y = 0.0011x - 0.0001. Results showed that the correlation coefficient (R2) was between 1.00 and 0.9981 (Table 1).

Table 1: Linearity data of BUDE and NAC by proposed UV and RP-HPLC Method

Parameters	UV Spectrophotometry		RP-HPLC	
	BUDE at 267 nm	NAC at 210 nm	BUDE at 216 nm	NAC at 216 nm
Linearity Range	0.24 - 1.2 µg/mL	20 to 100 µg/mL	0.24 - 1.2 µg/mL	20 to 100 µg/mL
Regression Equation	y = 0.0458x + 0.0001	y = 0.0011x - 0.0001	y = 1276.7x - 113.87	y = 27.054x + 204.27
Correlation Coefficient	1.00	0.9981	0.9998	0.9995
LOD	0.016	1.200	0.011	1.366
LOQ	0.048	3.636	0.032	4.141

Precision: In terms of precision, both Inter-day, Intraday and Repeatability measurements were conducted at three distinct concentrations 0.24, 0.48 & 0.72 µg/mL for BUDE and 20, 40 & 60 µg/mL for NAC in triplicate over three consecutive days and on the same day. The absorbance of the same solutions was measured. For repeatability, 0.48 µg/mL for BUDE and 40 µg/mL for NAC were measured. The resulting RSD values for Intraday, Inter-day precision, and Repeatability were showed in Table 2, respectively.

Table 2: Precision study of Budesonide & N-Acetylcysteine for UV Method

Intraday precision					
Conc. (µg/mL)		Mean Absorbance ±SD (n=3)		%RSD	
BUDE	NAC	BUDE	NAC	BUDE	NAC
0.24	20	|-0.011| ± 0.00013	|-0.021| ± 0.00024	1.19	1.12
0.48	40	|-0.022| ± 0.00022	|-0.041| ± 0.00039	0.99	0.95
0.72	60	|-0.033| ± 0.00024	|-0.064| ± 0.00044	0.73	0.68
Interday precision					
Conc. (µg/mL)		Mean Absorbance ±SD (n=3)		%RSD	
BUDE	NAC	BUDE	NAC	BUDE	NAC
0.24	20	|-0.010| ± 0.00012	|-0.022|±0.00026	1.22	1.16
0.48	40	|-0.021| ± 0.00021	|-0.042|±0.00041	1.04	0.98
0.72	60	|-0.032| ± 0.00025	|-0.065|±0.00047	0.78	0.72

Repeatability					
Conc. (µg/mL)		Mean Absorbance ±SD (n=3)		%RSD	
BUDE	NAC	BUDE	NAC	BUDE	NAC
0.48	40	|-0.023| ± 0.00024	|-0.042|±0.00040	1.04	0.97

LOD and LOQ: The minimum detectable quantity of an analyte within a sample by an analytical method was determined to be 0.016 µg/mL for BUDE at 267 nm and 1.2 µg/mL for NAC at 210 nm, The quantitation limit for a specific analytical method refers to the minimum quantity of the substance in a sample that can be accurately and precisely measured which was found to be 0.048 µg/mL for BUDE at 267 nm and 3.636 µg/mL for NAC at 210 (Table 1). The low LOD and LOQ values obtained at the selected wavelengths indicated the adequate sensitivity of the proposed UV spectrophotometric method for the estimation of both drugs.

Accuracy: To decide the accuracy of the technique recuperation, change into accomplished by means of standard addition approach. To pre-analysed pattern acknowledged quantity of general BUDE and NAC spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of BUDE and NAC. Accuracy was carried out by the Recovery Studies (standard addition method). The results were stipulated in triplicate and the accuracy indicated by % recovery. For UV, The % Recovery was obtained in range of 99.72%-99.95% for Budesonide and 99.87%-99.99% for N-Acetylcysteine were showed in Table 3.

Table 3: Recovery study data for UV and RP-HPLC Method

UV Method						
Name of Drug	% Level of recovery	Test Amount (µg/mL)	Amount of drug taken (µg/mL)	Total Std Amt (µg/mL)	Total amount Recovered (µg/mL)	% Mean Recovery ± SD(n=3)
Budesonide	50	0.48	0.24	0.72	0.718	99.72±0.011
	100	0.48	0.48	0.96	0.959	99.90±0.18
	150	0.48	0.72	1.2	1.197	99.95±0.48
N-Acetylcysteine	50	40	20	60	59.92	99.87±0.020
	100	40	40	80	79.99	99.98±0.038
	150	40	60	100	99.99	99.99±0.035
RP-HPLC Method						
Budesonide	50	0.48	0.24	0.72	0.719	99.86±0.20
	100	0.48	0.48	0.96	0.959	99.91±0.025
	150	0.48	0.72	1.2	1.199	99.98±0.010
N-Acetylcysteine	50	40	20	60	59.98	99.97±0.034
	100	40	40	80	79.99	99.98±0.010
	150	40	60	100	99.99	99.99±0.011

Assay as Analysis of Synthetic mixture: From assay, Final concentration of Budesonide was 0.48 µg/mL and N-Acetylcysteine 40 µg/mL were run into UV and The Percentage assay of Budesonide and N-Acetylcysteine were found to be 99.58 % and 99.95 %, respectively. Its results showed in Table 4.

Table 4: Assay as Analysis of synthetic mixture for UV and RP-HPLC Method

UV Method				
Name of Drug	Amount in synthetic mixture (µg/mL)	Mean Amount found (µg/mL)	% Assay ± SD (n=3)	%RSD
Budesonide	0.48	0.478	99.58 ± 0.428	0.429
N-Acetylcysteine	40	39.98	99.95 ± 0.071	0.0717
RP-HPLC Method				
Budesonide	0.48	0.479	99.79±0.02	0.021
N-Acetylcysteine	40	39.99	99.98±0.01	0.011

Validation Parameters of the RP-HPLC Method:

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Budesonide and N-Acetylcysteine shown in figure 10, 11 & 12.

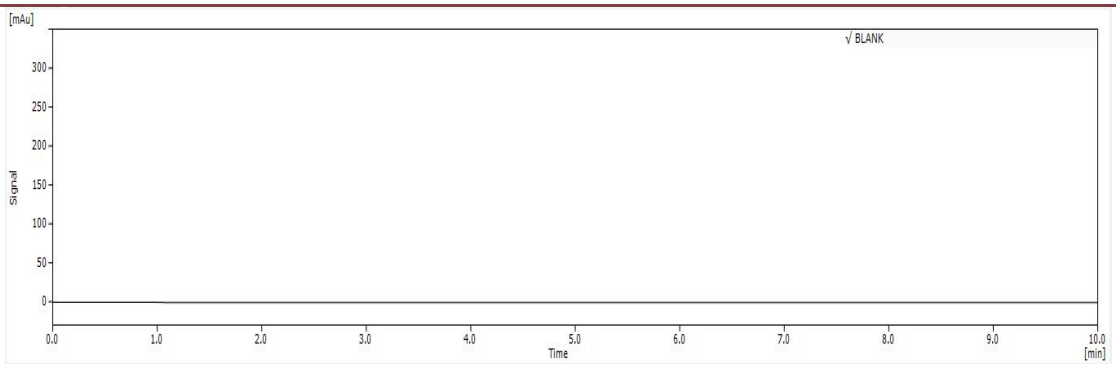

Figure 10: Blank RP-HPLC Chromatogram in Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) at 216 nm {Run time: 10 min, Flow rate: 1 ml/min}

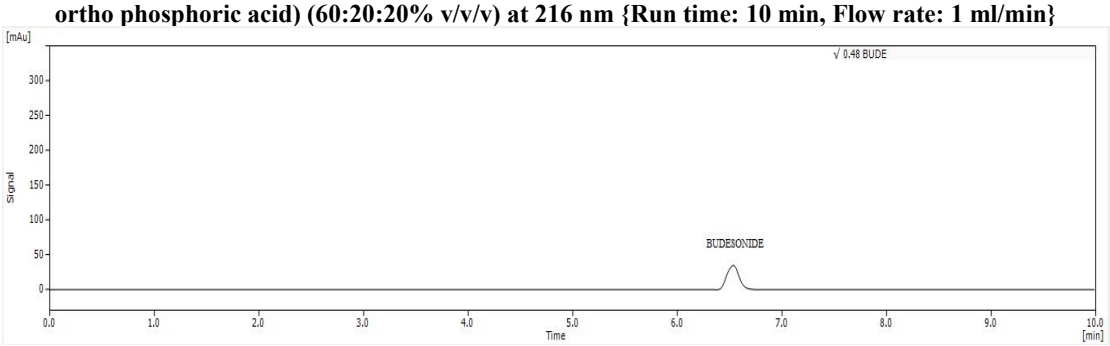

Figure 11: RP-HPLC Chromatogram of Budesonide (0.48 µg/mL) in Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) at 216 nm {Run time: 10 min, Flow rate: 1 ml/min}

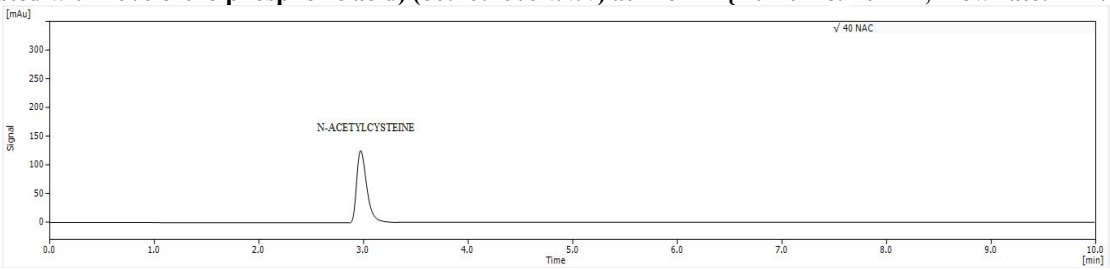

Figure 12: RP-HPLC Chromatogram of N-Acetylcysteine (40 µg/mL) in Methanol: Acetonitrile: Phosphate buffer (pH:3.5 adjusted with 10% ortho phosphoric acid) (60:20:20% v/v/v) at 216 nm {Run time: 10 min, Flow rate: 1 ml/min}

Linearity: The RP-HPLC chromatogram of N-Acetylcysteine (20-100 µg/mL) and Budesonide (0.24-1.2 µg/mL) at 216 nm showed in figure 13. The Peak Area was found. Linearity was showed in figure 13. Calibration graphs were plotted between concentrations and peak areas. The regression equation of calibration curve was generated y = 1276.7x - 113.87 for BUDE and y = 27.054x + 204.27 for NAC. The correlation coefficient (R2) values were observed to be 0.9998 and 0.9995. (Table 1).

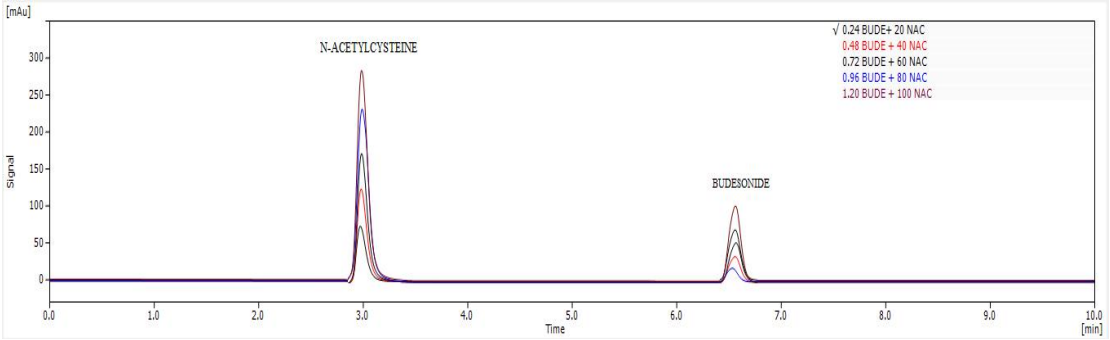

Figure 13: Overlain RP-HPLC chromatogram of N-Acetylcysteine (20-100 µg/mL) and Budesonide (0.24-1.2 µg/mL) at 216 nm {Run time: 10 min, Flow rate: 1 ml/min}

Precision: A concentration of 0.24, 0.48 & 0.72 µg/mL for BUDE and 20, 40 & 60 µg/mL for NAC. At same day three-time interval, the absorbance of the finished solution was measured in a 1.0 cm cell at a chosen wavelength. Likewise, on the first, second, and third days, the peak area of the same solutions was measured. Every solution is made in triplicate and examined. The resulting RSD values for Inter-day and Intraday precision were showed in table 5, respectively.

Table 5: Precision study for Budesonide & N-Acetylcysteine for RP-HPLC

Intraday precision		
Conc. (µg/ml)	**Mean peak area (mAu*sec) ± S.D (n=3)**	**%RSD**

BUDE	NAC	BUDE	NAC	BUDE	NAC
0.24	20	222.11±2.1544	782.74±7.9056	0.97	1.01
0.48	40	482.02±4.0007	1268.30±10.7805	0.83	0.85
0.72	60	790.51±5.4545	1778.90±12.4523	0.69	0.70
Interday precision					
Conc. (µg/ml)		**Mean Absorbance ±SD (n=3)**		**%RSD**	
BUDE	**NAC**	**BUDE**	**NAC**	**BUDE**	**NAC**
0.24	20	227.11±2.2938	784.74±8.3182	1.01	1.06
0.48	40	482.02±4.1453	1264.63±11.3816	0.86	0.90
0.72	60	790.51±5.8497	1781.23±13.5373	0.74	0.76
Repeatability					
Conc. (µg/ml)		**Mean Absorbance ±SD (n=3)**		**%RSD**	
BUDE	**NAC**	**BUDE**	**NAC**	**BUDE**	**NAC**
0.48	40	480.85±4.0872	1269.30±11.2967	0.85	0.89

Accuracy: The accuracy of the technique recuperation was decided change into accomplished by means of standard addition approach. To pre-analysed pattern acknowledged quantity of general BUDE and NAC spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of fashionable BUDE and NAC. The results were studied in triplicate and the accuracy changed into indicated by% recovery (Table 3). Accuracy was carried out by the Recovery Studies. For HPLC, The % Recovery was obtained in range of 99.86%-99.98% for Budesonide and 99.97%-99.99% for N-Acetylcysteine were showed in Table 3. The mean percentage recovery values for both drugs were found to be within the ICH-accepted range of 98-102%, with low standard deviation. These results confirm the accuracy, trueness, and reliability of the RP-HPLC method and indicated that excipients present in the synthetic mixture did not interfere with the estimation of either drug.

LOD and LOQ: LOD Values were found to be 0.011 and 1.366 µg/mL for Budesonide and N-Acetylcysteine, respectively. LOQ Values were found to be 0.032 and 4.141 µg/mL, respectively for Budesonide and for N-Acetylcysteine. These results showed in Table 1.

Assay: From assay, Final concentration of Budesonide was 0.48 µg/mL and N-Acetylcysteine 40 µg/mL were injected into HPLC System and The Percentage assay of Budesonide and N-Acetylcysteine were found to be 99.79 % and 99.98 %, respectively. Results showed in Table 4.

Robustness: Chromatographic analysis was used to analyse the effects of changes in analysts, and the results showed that there was no statistically significant difference in the% RSD of technique II. Additionally, small changes were performed to assess the robustness of the created HPLC procedures. The approaches' robustness was demonstrated by the% RSD, which remained constant despite minor variations in flow rate, run time, and detection. It was determined that the created approaches were essential as a result showed in Table 6.

Table 6: Robustness data

Condition	Variation	Budesonide	N-Acetylcysteine
		% Assay ± SD (n=3)	% Assay ± SD (n=3)
Flow rate (1 ml ± 0.2 ml/ min)	0.8 ml/min	98.98±2.3730	98.96±4.5166
	1.0 ml/min	99.95±3.5545	99.67±4.2691
	1.2 ml/min	99.75±5.0286	99.89±6.4770
Detection wavelength (216 nm ± 2 nm)	214	99.54±0.9454	99.65±6.1268
	216	100.05±1.5055	100.01±6.4267
	218	99.95±1.3762	99.93±7.0256
Mobile Phase Methanol: Acetonitrile: Phosphate buffer (pH:3.5) (60:20:20 ± 2 % v/v/v)	58:18:24	99.75±2.1116	99.26±2.0784
	60:20:20	99.85±2.0552	99.92±1.9421
	62:22:16	98.99±2.1845	99.95±2.0143

Statistical Evaluation of Analytical Methods: A statistical approach was used to differentiate between the proposed analytical approaches. There were no discernible differences among the quantities measured acquired in the subject matter evaluation through the two separate procedures based on student t-test findings. At the 5% significance level, the estimated t-value (from formula) proved less than the critical t-value (from the statistics database) and it was found that the tabulated values were greater than calculated values. So, there was no significant difference between Recovery and Assay parameters obtained through U.V. method and RP-HPLC method. Results indicated that both developed and validated analytical methods were considered accurate, precise, and statistically insignificant. The results of Compared Recovery and assay data to compare Between U.V. and RP-HPLC Methods were showed in table 7.

Table 7: Statical Comparison data of developed methods by Student t-test Analysis

Result of Compared Recovery Data

t-test Value (Between U.V. and RP-HPLC Method)	Budesonide	N-Acetylcysteine
T calculated	0.76	0.68
T tabulated	2.12	2.12
t-test at 95% confidence interval ($p \leq 0.05$ and d.f. = 16)		
Result of Compared Assay Data		
t-test Value (Between U.V. and RP-HPLC Method)	Budesonide	N-Acetylcysteine
T calculated	0.17	0.39
T tabulated	2.78	2.78
t-test at 95% confidence interval ($p \leq 0.05$ and d.f. = 4)		

CONCLUSION

The UV spectrophotometry and RP-HPLC methods were linear, precise, accurate, and validated in compliance with ICH Q2 (R2) guideline. The RP-HPLC and UV spectrophotometric technique have been developed and validated for routine measurement of BUDE and NAC in laboratory-prepared synthetic mixtures. These techniques are easy to use, fast, accurate, and precise. Without interfering, all two methods may determine BUDE and NAC concurrently in multi-component. Overall, the validated first-order derivative UV-spectrophotometric and RP-HPLC method were simple, precise, accurate, sensitive, and cost-effective, making it suitable for routine quality control analysis of BUDE and NAC in synthetic mixtures. The established protocols should be followed for routine and quality control drug analysis in pharmaceutical compositions with two components. The low standard deviation and percentage RSD values of the suggested techniques make them appropriate for regular BUDE and NAC measurements in laboratory-prepared synthetic mixtures. The statistical results indicated that both methods were equally sensitive, reliable, and can routinely apply for simultaneous estimation of Budesonide and N-Acetylcysteine in Synthetic Mixture.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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