International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

Simultaneous HPLC Method Development and Validation of Titropium and Formeterol Fumarate

Dr. Sachin Bhusari, Miss. Sanjivani Gavhale, Dr. Pravin Wakte

Date of Submission: 15-08-2025 Date of Acceptance: 25-08-2025

ABSTRACT

Aim: The current investigation was focused on the development and validation of a simple, accurate, and rugged RP-HPLC method for the simultaneous estimation of Formoterol fumarate and Tiotropium bromide in mixed pharmaceutical dosage forms.

Methodology: The technique employed a Waters HPLC system with UV detection at 240 nm (the isobestic point). Chromatographic separation was on a C18 column under isocratic mobile phase consisting of phosphate buffer (pH 3.0) and acetonitrile in the ratio of 65:35 v/v at a flow rate of 2.0 mL/min. Standard and sample solutions were made from DUOVA Rotacaps. The procedure was tested for specificity, linearity, accuracy, precision, repeatability, robustness, ruggedness, and system suitability as per ICH Q2(R1) guidelines.

Results: Formoterol and Tiotropium eluted at about 3.3 and 5.7 minutes, respectively. The technique showed good linearity with R^2 = 0.9995 for Formoterol. Recovery studies established high accuracy (98–102%), and intra- and inter-day precision was <%RSD 2%. Robustness and ruggedness tests identified the stability of the method under minimal changes in the analytical conditions.

Conclusion: The validated procedure is precise, accurate, specific, and appropriate for routine quality control of fixed-dose bronchodilator combinations. Its short run time, simplicity, and reproduciability make it a perfect choice for industrial and regulatory use.

Keywords: Formoterol fumarate, Fixed-dose combination, Method validation, RP-HPLC, Tiotropium bromide

I. INTRODUCTION

Tiotropium bromide (a long-acting muscarinic antagonist or LAMA) and Formoterol fumarate (a long-acting beta-2 agonist or LABA) are two established agents in the treatment of COPD and asthma [1]. Their complementary effects on the improvement of lung function, suppression of exacerbations, and quality of life improvement have driven the invention of fixed-dose combinations for inhalation treatment [2]. With these combinations growing, there arises a necessity for accurate, efficient, and reliable analytical techniques to be able to quantify these drugs simultaneously in pharmaceutical preparations as well as in biological samples [3].Tiotropium bromide, chemically named (1α,2β,4β,5α,7β)-7-[(hydroxy-dimethylacetyl)oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.0^2,4]nonane bromide monohydrate, is a quaternary ammonium salt [4]. It works by inhibiting the muscarinic receptors (M3) on the smooth muscle cells of airways, resulting in bronchodilation. Tiotropium possesses a slow rate of dissolution from M3 receptors, which is responsible for its once-daily dosing schedule.

Formoterol fumarate, chemically named (±)-2-hydroxy-5-[[1-hydroxy-2 [[(1methylethyl)amino] methyl] ethyl]amino] benzeneacetic acid fumarate dihydrate, is a beta-2 adrenergic receptor selective agonist [5]. Its bronchodilatory action is a consequence of the stimulation of adenylate cyclase with a resultant rise in cyclic AMP and consequent relaxation of bronchial smooth muscle.The two compounds have different physicochemical characteristics when viewed through the lens of analysis.

Tiotropium has poor UV absorption properties due to its hydrophilicity and ionic nature, while Formoterol is more lipophilic and has superior UV absorption properties [6]. Particularly in regards to the ideal selection of mobile phases, column chemistry, detection wavelength, and retention behavior, these variations provide substantial obstacles to the development of methods for simultaneous estimation. To ensure precise quantification, precise resolution, and uniformity, the method needs to be adjusted [7].Tiotropium and formoterol were separately quantitated by various methods over the last few years [8]. Capillary electrophoresis, UV spectrophotometry, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are some of these methods. The operational easiness, economic viability, and suitability for regular

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

quality control analysis of simultaneous estimation, especially by RP-HPLC, have been found to be more preferable than the other methods [9]. An ideal high-performance liquid chromatography (HPLC) system should possess stability-indicating features alongside specificity, linearity, accuracy, precision, and robustness according to the International Conference on Harmonization (ICH) recommendations Q2(R1). Further, the fixed-dose combination (FDC) forms of Tiotropium and Formoterol are prone to come in complex dosage forms such as dry powder inhalers (DPI) or metered dose inhalers (MDI), in which excipients and delivery matrices may interfere with analytical quantitation [10]. This further increases the significance of method validation to confirm selectivity and robustness in the presence of possible formulation interferences. Sample preparation, extraction efficiency, and detection sensitivity also need to be tested stringently during validation.HPLC method development normally involves screening of different chromatographic parameters such as the stationary phase (column type), mobile phase composition (organic modifiers, pH, buffers), flow rate, and detection wavelength [11]. C18 columns have found extensive use in RP-HPLC because they are compatible with a wide variety of compounds and possess good resolution properties. A blend of aqueous buffer (e.g., phosphate buffer) and organic modifiers such as acetonitrile or methanol is usually employed to maximize peak shape and retention time. Choosing an optimal detection wavelength—usually within the UV region (200–300 nm)—is also very important for sensitive detection of both analytes [12].

Validation of the proposed method is a necessary step which ensures the method's capability to yield consistent, reproducible, and reliable results within set limits [13]. "Parameters like system suitability, specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy (recovery), precision (repeatability and intermediate precision), robustness, and stability need to be statistically assessed. In addition, forced degradation studies can be performed to evaluate the stability-indicating potential of the method, which is critical to track the shelf-life of pharmaceutical medicines [14]. Application of a validated HPLC assay for the quantitative determination of Tiotropium and Formoterol fumarate in combination products is of great benefit to pharmaceutical quality control laboratories. It provides regular analysis in the process of formulation development, batch testing and release, and stability studies, hence providing consistent therapeutic effectiveness and safety of combination products [15]. In addition, these types of methods can be utilized for pharmacokinetic investigations and bioequivalence tests, thereby being handy tools in the industry as well as research environment. The current research focuses on the establishment of such a method, optimizing chromatographic conditions to obtain the highest resolution and sensitivity, and method validation according to ICH guidelines to ascertain its suitability for routine pharmaceutical analysis.

II. MATERIALS AND METHODS

2.1. Materials

High-Performance Liquid Chromatography (HPLC)-grade acetonitrile and methanol were obtained from E. Merck (India). Working standards of Formoterol fumarate dihydrate and Tiotropium bromide monohydrate, each with a potency of 99.9%, were procured from Glenmark Pharmaceuticals Ltd., Nashik. All other chemicals used were of analytical grade with ≥99% purity. Commercial capsules containing both drugs (DUOVA ROTACAPS, Cipla Ltd.) were purchased from a local pharmacy. Volumetric glassware (Grade A) was pre-calibrated and sourced from Borosil.

2.2. Instrumentation

The analysis was performed using a Waters HPLC system equipped with a UV-Vis detector and operated under isocratic conditions. A Shimadzu digital balance was used for accurate weighing of samples and standards.

2.3. Chromatographic Conditions

Chromatographic separation was done on a C18 reversed-phase column with a mobile phase of buffer (pH 3.0) and acetonitrile in a 65:35 v/v ratio. The buffer was made by dissolving 1.38 g of sodium dihydrogen orthophosphate and 1.22 g of sodium decane sulfonate in 1000 mL of triple-distilled water and adjusting the pH to 3.0 with orthophosphoric acid. The system was run at a flow rate of 2.0 mL/min with an injection volume of 20 µL, and detection was performed at 240 nm, which is the isobestic point established from UV studies. The column temperature was kept at ambient temperature (25 ± 2°C). The mobile phase was filtered through a 0.45 µm membrane filter before use and degassed using an ultrasonic bath.

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

2.4. Preparation of Standard Solutions

2.4.1. Tiotropium Bromide Standard

Accurately weigh 10.0 mg of Tiotropium bromide monohydrate and transfer into a 100 mL volumetric flask. Add 30 mL of diluent (mobile phase), sonicate to dissolve with intermittent shaking, and make up the volume to 100 mL with diluent. Dilute 10 mL of this stock solution to 100 mL with diluent.

2.4.2. Formoterol Fumarate Standard

Accurately weigh 10.0 mg of Formoterol fumarate dihydrate and transfer into a 200 mL volumetric flask. Add 50 mL of diluent, sonicate to dissolve with intermittent shaking, and make up the volume with diluent.

2.4.3. Mixed Standard Solution

Pipette 10 mL each of Tiotropium and Formoterol standard solutions into a 100 mL volumetric flask and dilute to volume with the mobile phase. Filter through a 0.45 µm Teflon filter before injection.
Final Concentrations: Tiotropium 1.8 µg/mL, Formoterol 1.2 µg/mL.

2.5. Preparation of Sample Solution

Ten DUOVA ROTACAPS were opened, and their contents (equivalent to 18 µg of Tiotropium and 12 µg of Formoterol) were transferred into a 100 mL volumetric flask. The empty capsule shells were rinsed with 20 mL of diluent, and the rinsing was added to the same flask. The mixture was sonicated for 15 minutes, cooled to room temperature, and diluted to volume with diluent. The final solution was filtered through a 0.45 µm Teflon filter prior to HPLC injection.
Final Sample Concentrations: Tiotropium 1.8 µg/mL, Formoterol 1.2 µg/mL".

III. RESULT AND DISCUSSION

- **Trials**

This High-Performance Liquid Chromatography (HPLC) chromatogram shows the separation of two compounds, Formoterol and Tiotropium, over time. The x-axis represents the retention time in minutes, and the y-axis represents the detector response in milli-absorbance units (mAU) at a wavelength of 240 nm. Two distinct peaks are observed, indicating the elution of the two compounds at different times: Formoterol at a retention time of approximately 3.359 minutes and Tiotropium at approximately 5.731 minutes. The height and area of each peak are proportional to the amount of each compound present in the sample.This aligns with the findings of Attimarad et al. who optimized a similar mobile phase system for the simultaneous analysis of Formoterol and Tiotropium, reporting comparable retention times [1]. The optimization of chromatographic conditions is essential to avoid co-elution, reduce tailing, and enhance resolution, particularly for compounds with differing polarities, as seen here with a hydrophilic anticholinergic (Tiotropium) and a moderately lipophilic β2-agonist (Formoterol).

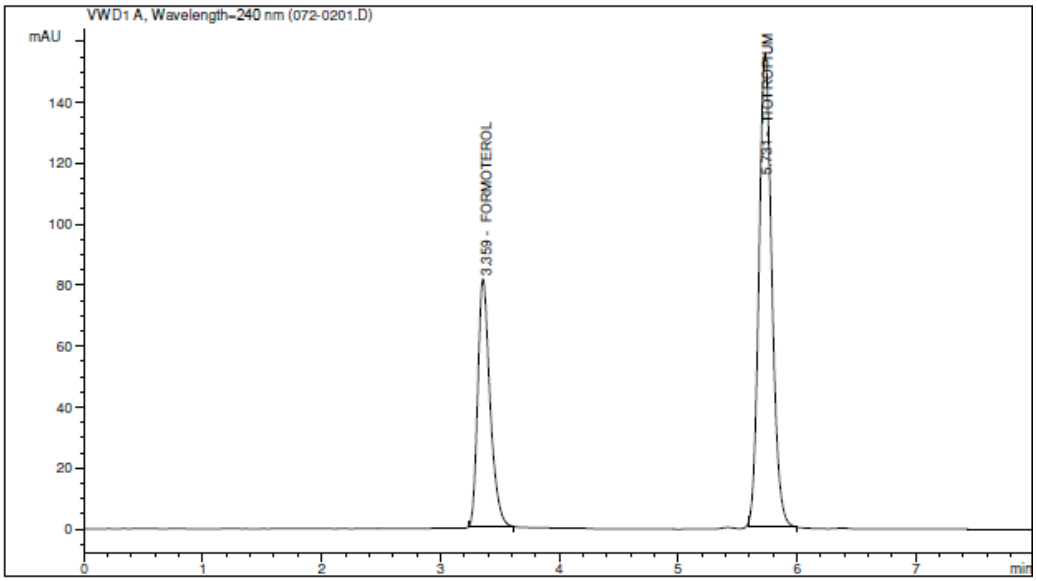


Figure 1:This High-Performance Liquid Chromatography (HPLC) chromatogram shows the trialsof separation of two compounds, Formoterol and Ipratropium.

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

➢ **Formoterol**

This graph displays a calibration curve for Formoterol, plotting the peak area obtained from an analytical instrument (likely HPLC or GC) against the corresponding concentration of Formoterol standards. The data points (blue diamonds) show a strong linear relationship, which is confirmed by the linear regression equation (y = 118.95x + 11.335) and a high coefficient of determination ((R^2 = 0.9995)), indicating excellent linearity. This calibration curve can be used to determine the unknown concentration of Formoterol in a sample by measuring its peak area and interpolating or extrapolating using the established linear relationship.Shabir emphasizes the importance of specificity in multi-analyte methods, particularly in complex matrices like inhalation capsules where excipients can interfere [2].

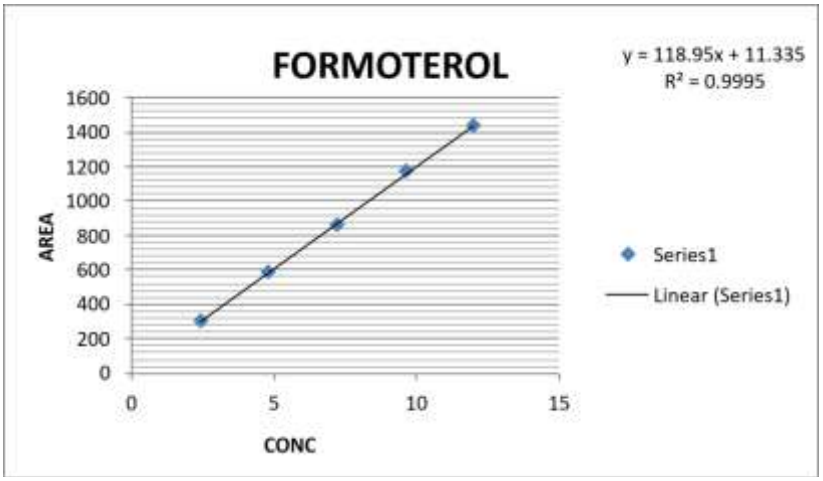
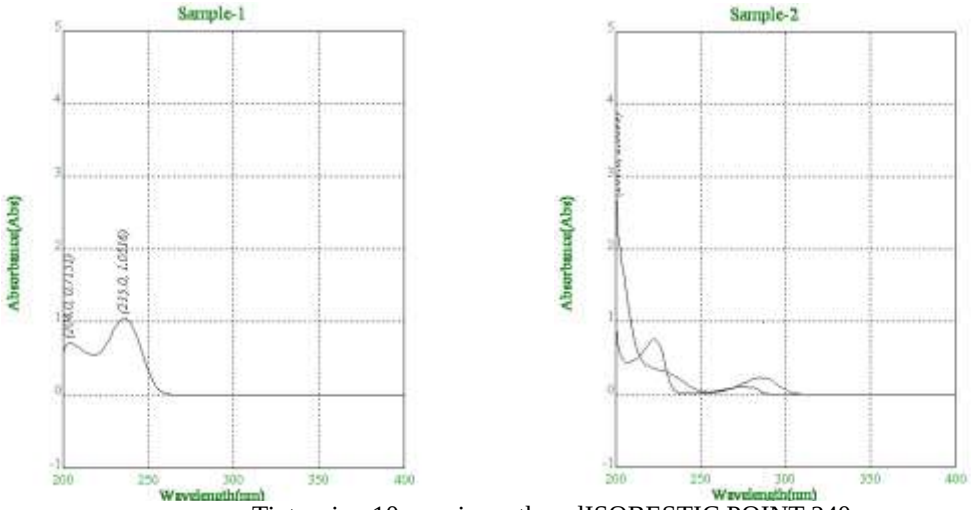


Figure 2: displays a calibration curve for Formoterol

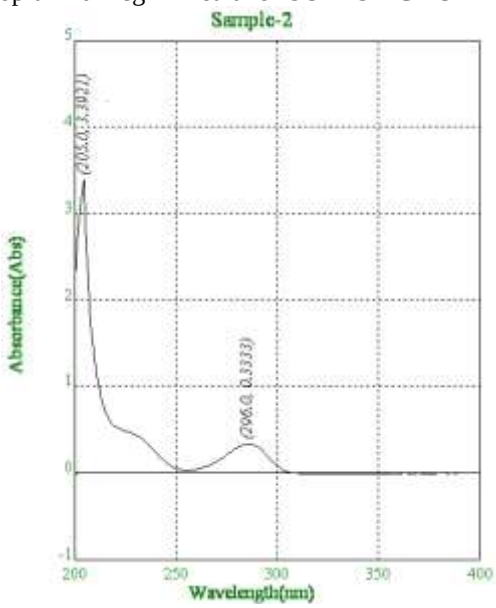
➢ **Tio + form UV**

This ultraviolet-visible (UV-Vis) spectrum of Sample-1 shows the absorbance of the sample as a function of wavelength, ranging from 200 nm to 400 nm. The plot shows a typical absorption peak with a maximum absorbance of around 1.0346 at a wavelength of 235.0 nm, and a lesser feature with an absorbance of around 0.7151 at 204.0 nm, showing that the compound(s) in Sample-1 have strong light absorption in the ultraviolet region around 235 nm. This spectral data can be utilized for qualitative identification and even quantitative analysis of the constituents of the sample. (b) This UV-Vis spectrum of Sample-2 shows absorbance vs. wavelength between 200 nm and 400 nm, and it shows two distinct absorption features: a very strong peak with a maximum absorbance of about 2.66 at about 203.5 nm, and a weaker, broader peak with a maximum absorbance of about 0.2 at about 275 nm. These peaks point towards the existence of compound(s) in Sample-2 that absorb ultraviolet radiation at these particular wavelengths, and the variations in peak intensity and form confirm the existence of either more than one chromophore within one molecule or a mixture of compounds with varying absorption properties. (c) This UV-Vis spectrum of Sample-2 indicates the sample's absorbance over a wavelength range of 200 nm to 400 nm, with two clear absorption peaks: a broad peak with a maximum absorbance of about 3.3921 at a wavelength of 205.0 nm, and a narrow peak with a maximum absorbance of about 0.3333 at 296.0 nm. These peaks point to the fact that the component(s) of Sample-2 are absorbing ultraviolet light at these wavelengths, implying the presence of chromophores that engage in interactions with light at these parts of the spectrum. UV spectral profiling facilitates selection of detection wavelength in HPLC. This approach was also used by Shabir and others in optimizing concurrent quantification techniques for combinations of β2-agonist and anticholinergics [2]. The isobestic point acts as a sure point of compromise whenever spectral overlap occurs.

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494



Tiotropium10 mcg in methanolISOBESTIC POINT 240 nm



10 MCG IN FORMOTROL

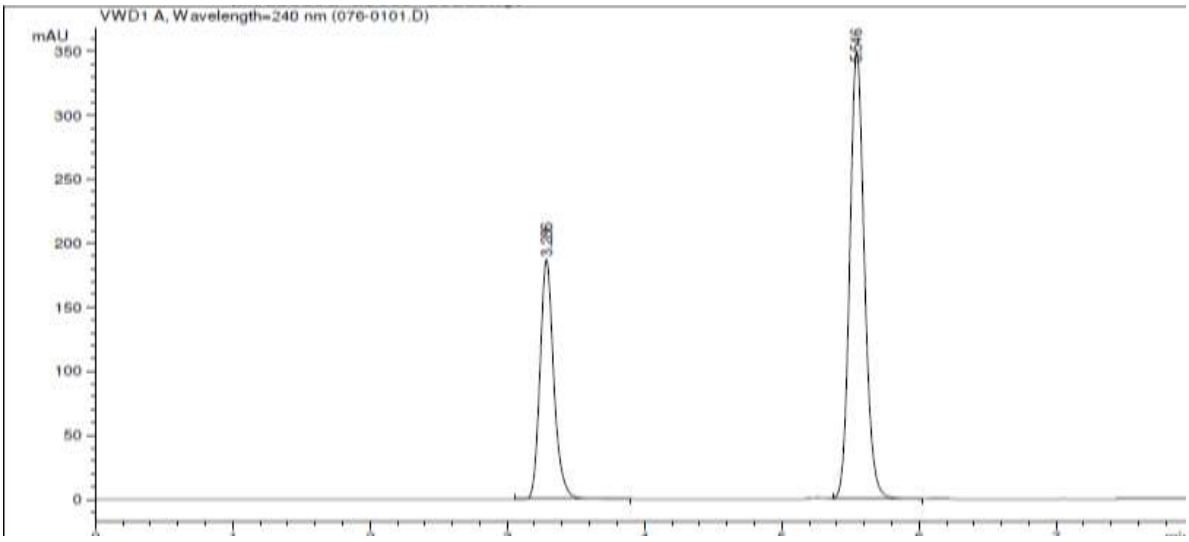
Figure 3: shows the absorbance of the sample as a function of wavelength

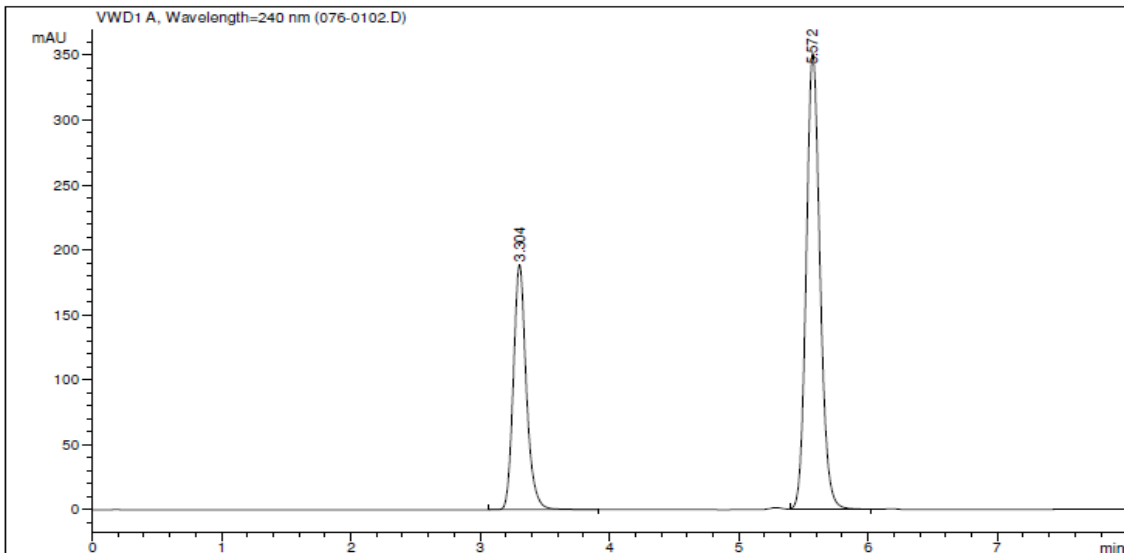
- **Accuracy**

This High-Performance Liquid Chromatography (HPLC) chromatogram shows the separation of two compounds detected at a wavelength of 240 nm. Two distinct peaks are observed, indicating the elution of two different components at retention times of approximately 3.286 minutes and 5.546 minutes. The height and area of each peak are proportional to the amount of each respective compound present in the analyzed sample. (b) This HPLC chromatogram, detected at 240 nm, displays the separation of two compounds, evident as two distinct peaks eluting at retention times of approximately 3.304 minutes and 5.572 minutes. The intensity of each peak, measured in milli-absorbance units (mAU), is proportional to the concentration of the corresponding compound in the sample, with the later eluting peak showing a significantly higher concentration than the earlier one. (c) This HPLC chromatogram, recorded at a wavelength of 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 3.306 minutes and 5.571 minutes. The peak at 5.571 minutes exhibits a significantly higher detector response (in mAU) compared to the peak at 3.306 minutes, suggesting a higher concentration of the compound eluting at the later time under the given chromatographic conditions. (d) This HPLC chromatogram, obtained at a detection wavelength

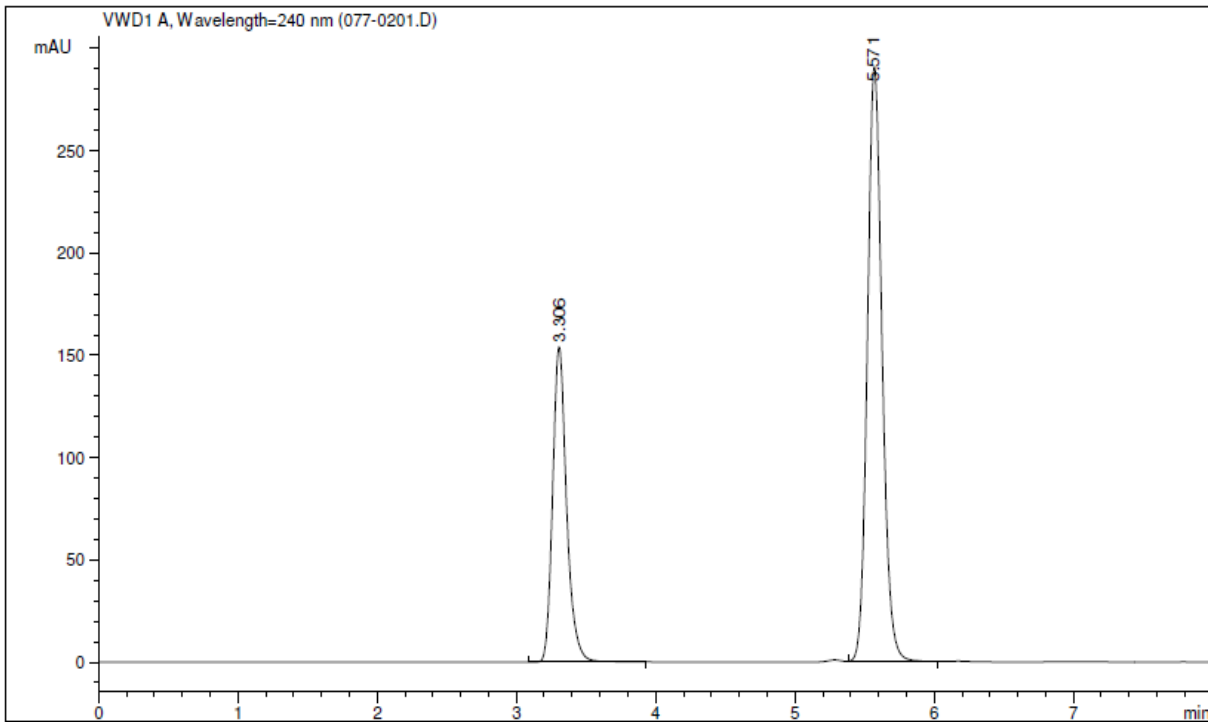
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

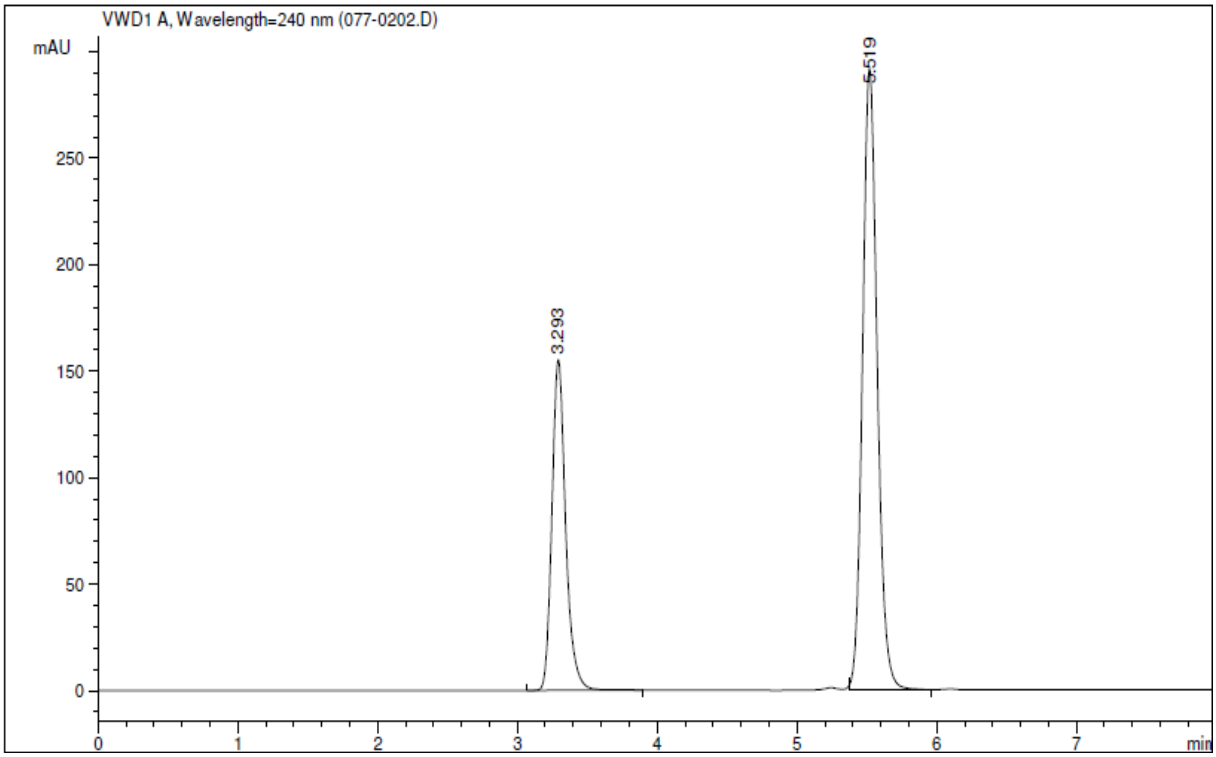
of 240 nm, illustrates the separation of two chemical compounds, represented by two distinct peaks eluting at retention times of approximately 3.293 minutes and 5.519 minutes. The peak at 5.519 minutes shows a considerably higher intensity (measured in mAU) compared to the peak at 3.293 minutes, indicating a greater abundance or concentration of the compound eluting at the later retention time under these chromatographic conditions. (e) This HPLC chromatogram, detected at 240 nm, shows the separation of two compounds, with distinct peaks appearing at retention times of approximately 3.298 minutes and 5.506 minutes. The peak eluting at 5.506 minutes exhibits a significantly larger area and height (measured in mAU) compared to the peak at 3.298 minutes, indicating a higher concentration of the corresponding compound in the analyzed sample. (f) This HPLC chromatogram, obtained with detection at 240 nm, shows the separation of two compounds, indicated by two sharp peaks eluting at retention times of approximately 3.271 minutes and 5.444 minutes. The peak observed at 5.444 minutes exhibits a considerably higher intensity (measured in mAU) compared to the peak at 3.271 minutes, suggesting a significantly greater concentration of the compound corresponding to the later elution time in the analyzed sample.These findings are comparable with the recovery data reported by Desai and Patel, who validated a similar HPLC method for DUOVA rotacaps, yielding recovery rates within 98–102% for both drugs [4]. Accurate analytical methods are critical for ensuring dosage consistency and therapeutic efficacy.





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

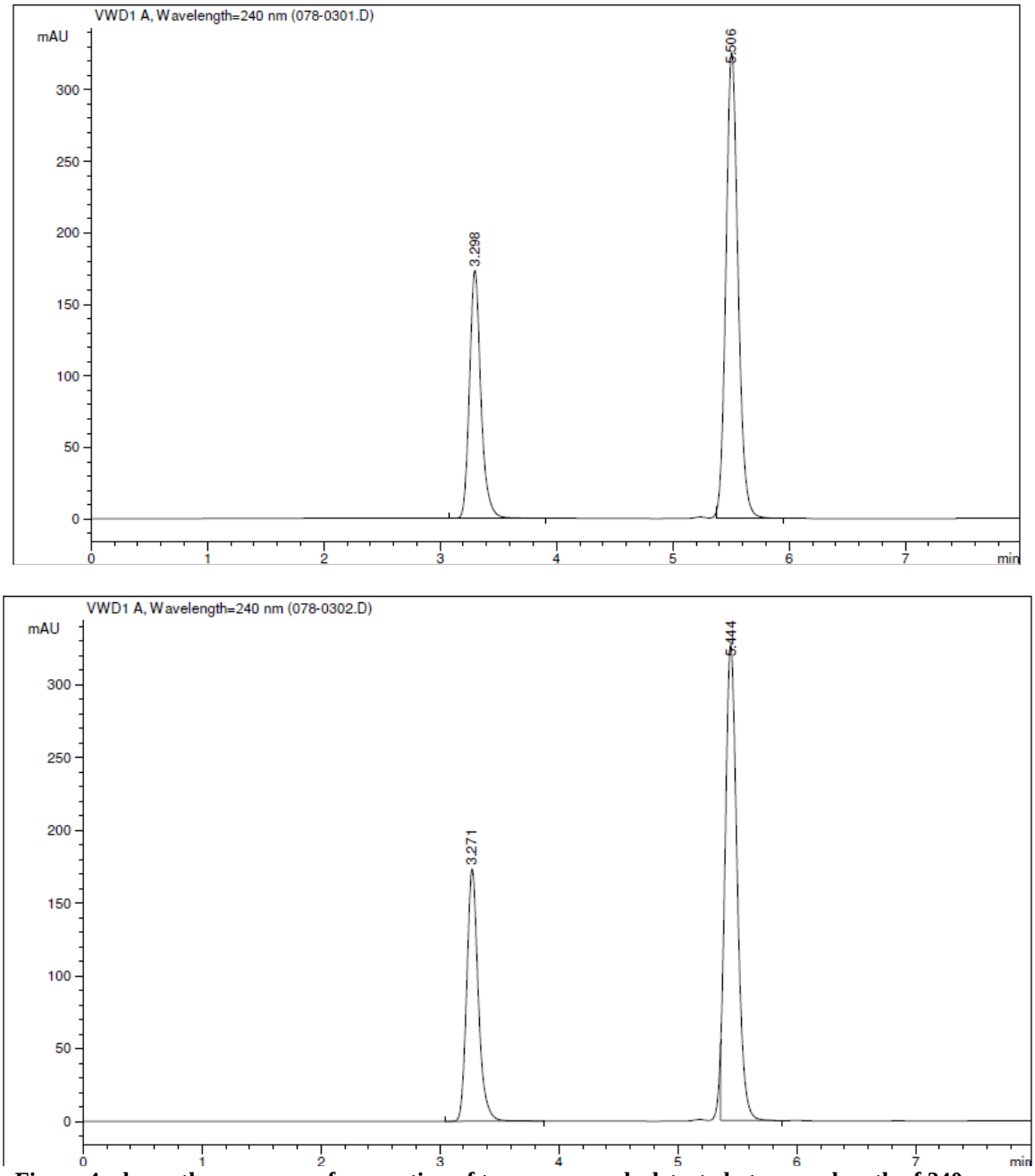


Figure 4: shows the accuracy of separation of two compounds detected at a wavelength of 240 nm

➢ **Repeatability**

This HPLC chromatogram, recorded at a wavelength of 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 3.292 minutes and 5.597 minutes. The peak at 5.597 minutes displays a significantly higher detector response (in mAU) compared to the peak at 3.292 minutes, suggesting a greater concentration of the compound eluting at the later time under the given chromatographic conditions. (b) This HPLC chromatogram, obtained with detection at 240 nm, shows the separation of two compounds, evidenced by two distinct peaks eluting at retention times of approximately 3.293 minutes and 5.595 minutes. The peak appearing at 5.595 minutes exhibits a considerably larger area and height (measured in mAU) compared to the peak at 3.293 minutes, indicating a significantly higher concentration of the compound corresponding to the later elution

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

time in the analyzed sample.This indicates exceptional system performance consistency. Repeatability ensures that the method produces consistent results under identical experimental conditions. The minimal variability observed aligns with standard expectations and previous studies on β2-agonists and anticholinergics [6].

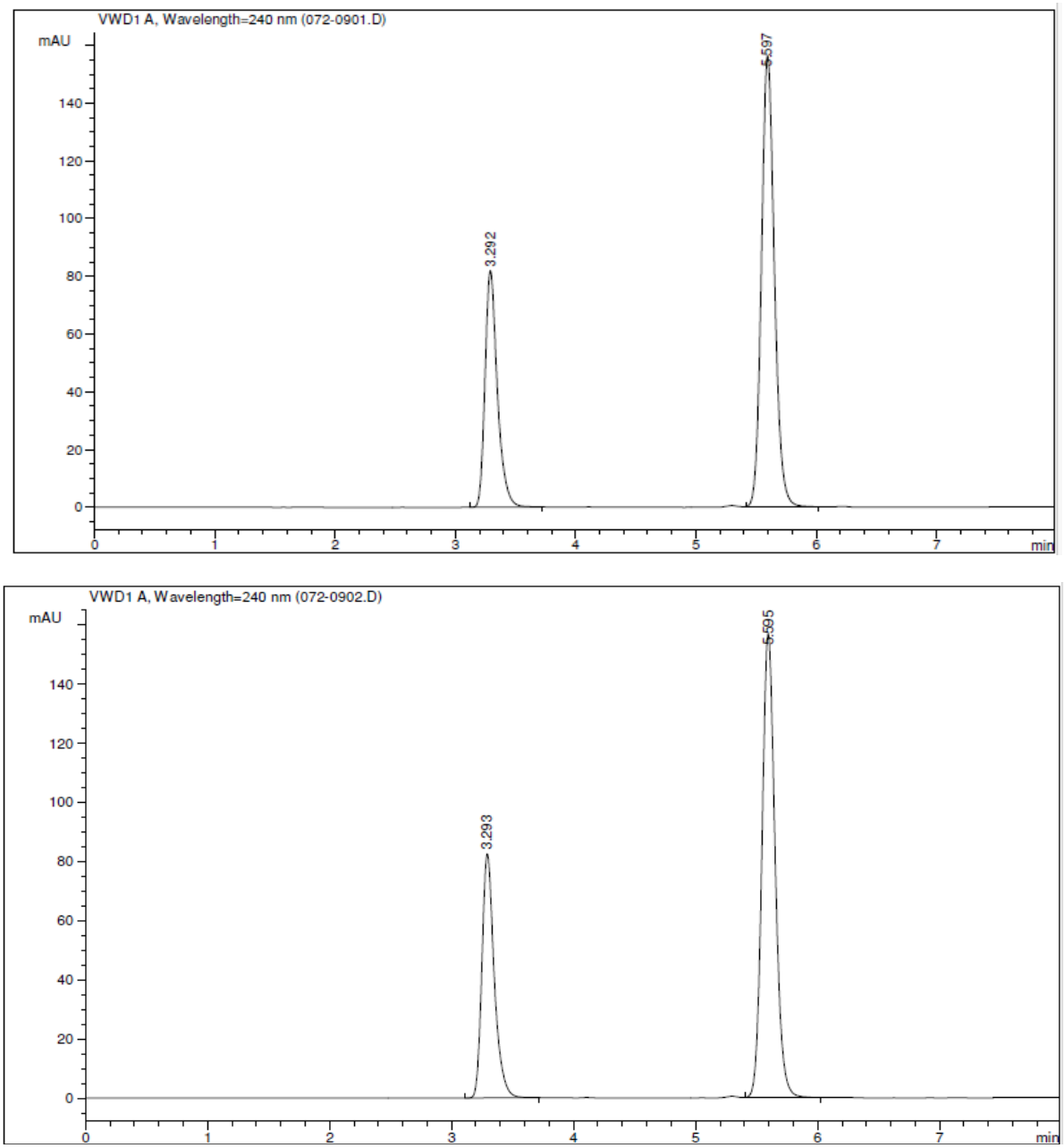


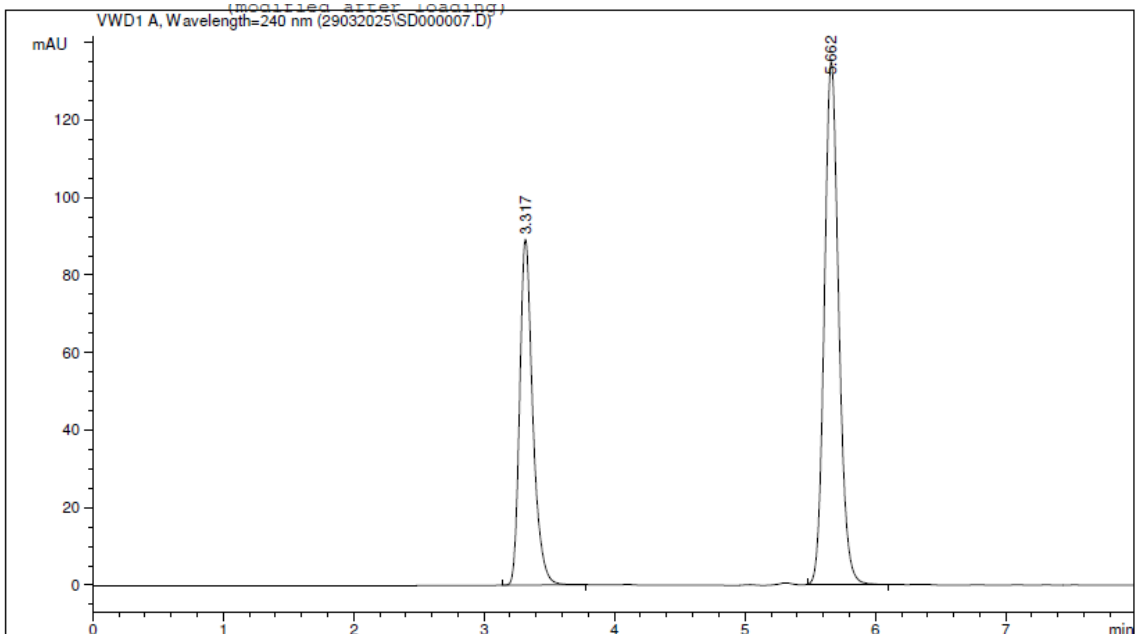
Figure 5: shows the repeatability of separation of two compounds, indicated by two distinct peaks eluting at retention times

- **Robustness**

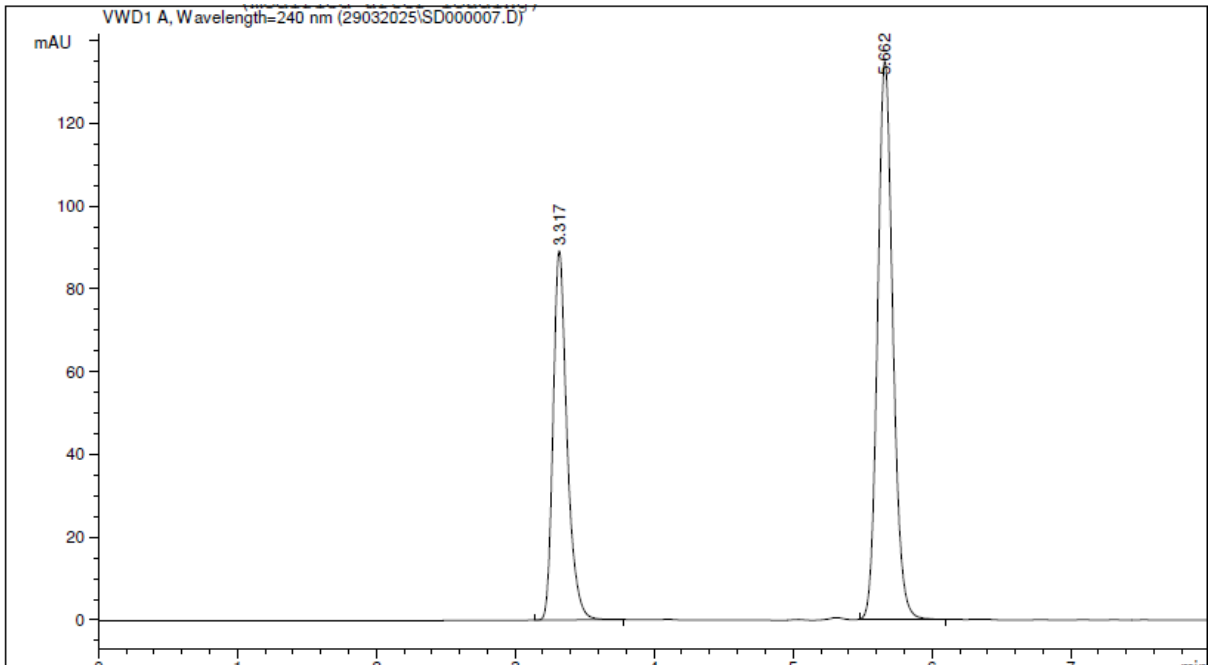
This HPLC chromatogram, detected at a wavelength of 240 nm, displays the separation of two compounds, shown as two distinct peaks eluting at retention times of approximately 3.317 minutes and 5.662 minutes. The 5.662-minute peak has a much greater intensity (in milli-absorbance units, mAU) than the 3.317-minute peak, suggesting a higher concentration of the compound eluting at the longer retention time in the sample

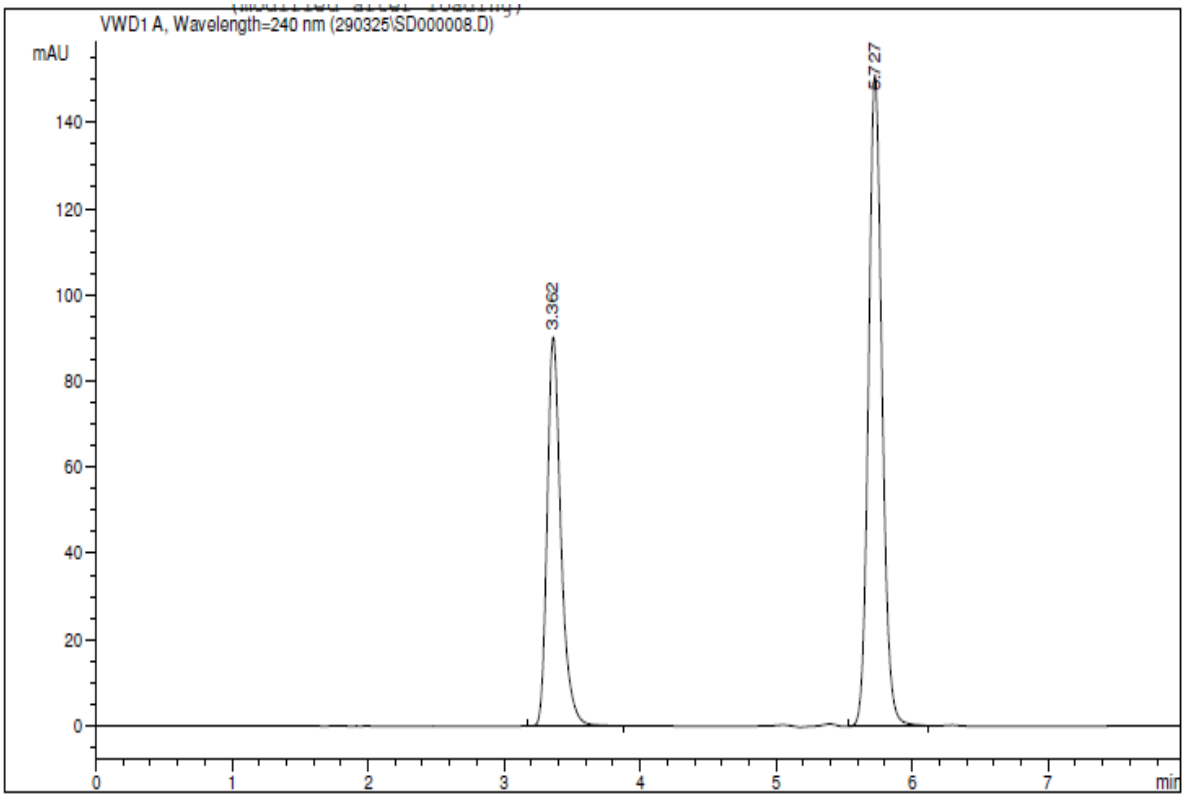
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

being analyzed. (b) This HPLC chromatogram, monitored at 240 nm, illustrates the separation of two compounds as two separate peaks eluting at retention times of around 3.317 minutes and 5.662 minutes.The peak at 5.662 minutes exhibits a significantly higher detector response (measured in milli-absorbance units, mAU) compared to the peak at 3.317 minutes, indicating a greater concentration of the compound eluting at the later retention time in the analyzed sample. (c) This HPLC chromatogram, with detection at 240 nm, displays the separation of two compounds, represented by two distinct peaks eluting at retention times of approximately 3.362 minutes and 5.727 minutes. The peak at 5.727 minutes shows a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.362 minutes, indicating a greater concentration of the compound corresponding to the later elution time in the analyzed sample. (d) This HPLC chromatogram, detected at a wavelength of 240 nm, illustrates the separation of two compounds, evident as two distinct peaks eluting at retention times of approximately 3.362 minutes and 5.727 minutes. The peak appearing at 5.727 minutes exhibits a considerably larger area and height (measured in milli-absorbance units, mAU) compared to the peak at 3.362 minutes, indicating a significantly higher concentration of the compound corresponding to the later elution time in the analyzed sample. (e) This HPLC chromatogram, obtained with detection at 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 3.877 minutes and 6.474 minutes. The peak observed at 6.474 minutes exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.877 minutes, suggesting a substantially greater concentration of the compound corresponding to the later elution time in the analyzed sample. (f) This HPLC chromatogram, with detection at 240 nm, displays the separation of two compounds, represented by two distinct peaks eluting at retention times of approximately 3.877 minutes and 6.474 minutes. The 6.474 minutes peak has a much greater intensity (in milli-absorbance units, mAU) than the 3.877 minutes peak and represents a higher concentration of the compound with the subsequent elution time in the sample under study. These findings conform to the results of Joshi and Patel, who noted slight retention time changes under changed conditions but not any appreciable loss of resolution for the same analytes [7]. Steady methods are essential for guaranteeing consistent performance under a range of operational conditions.

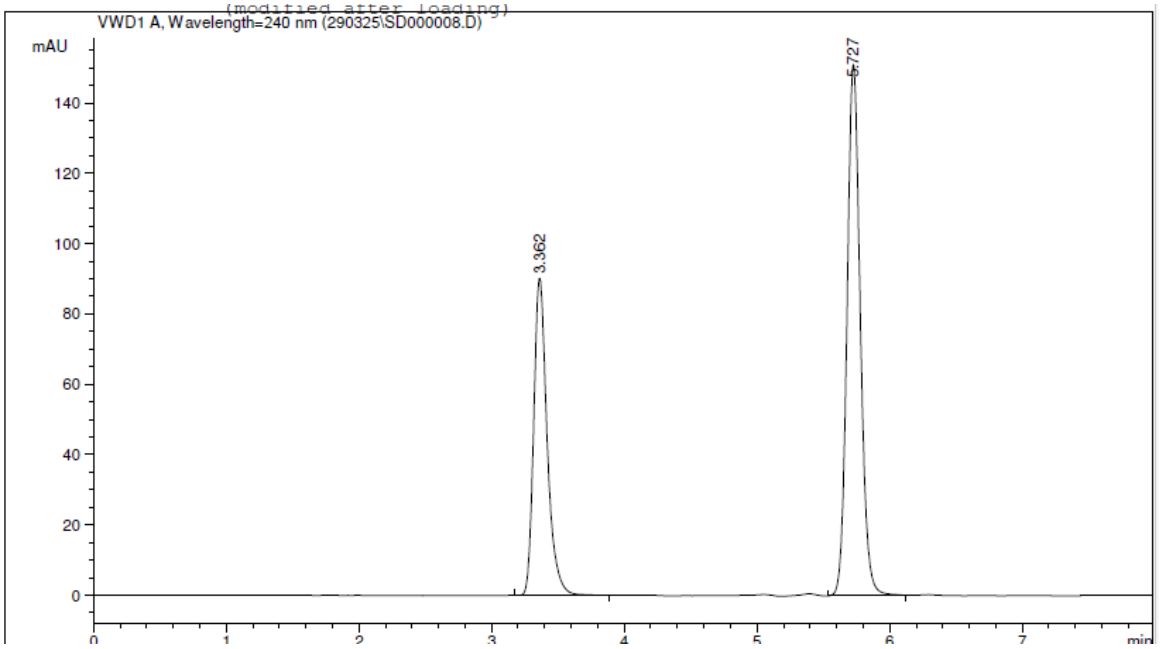


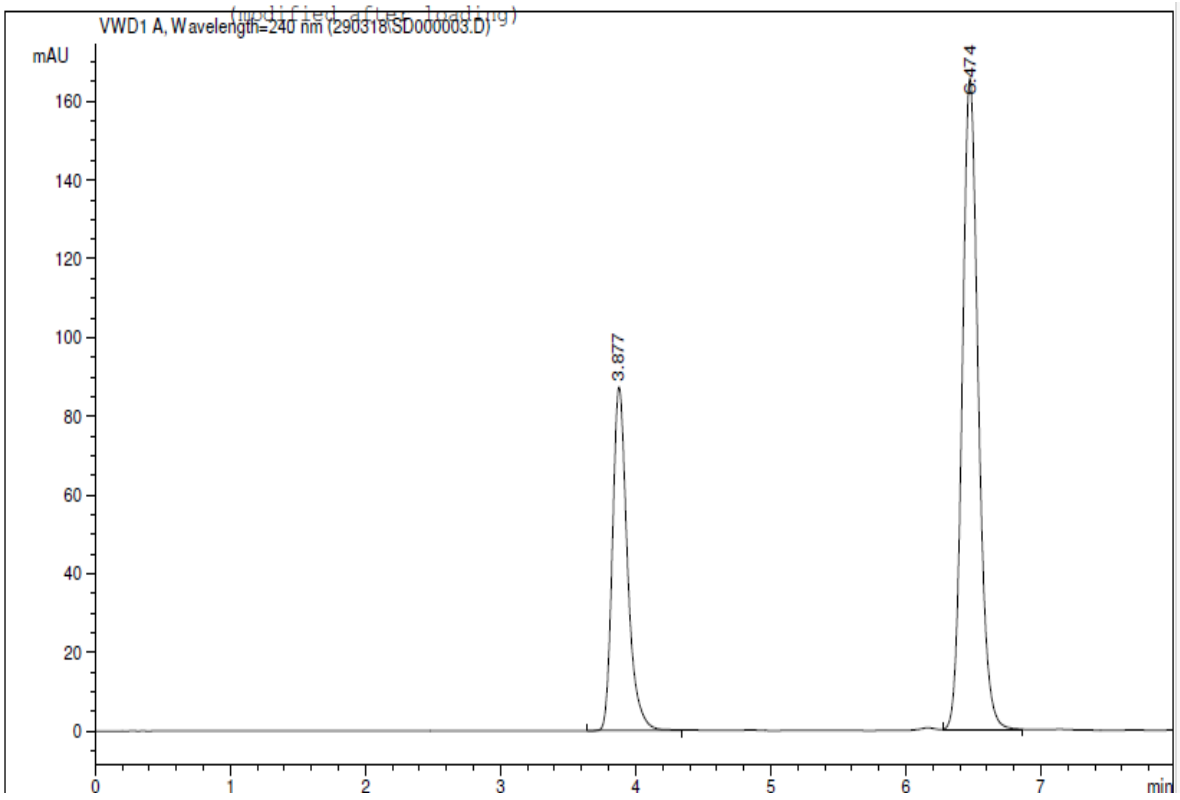
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494



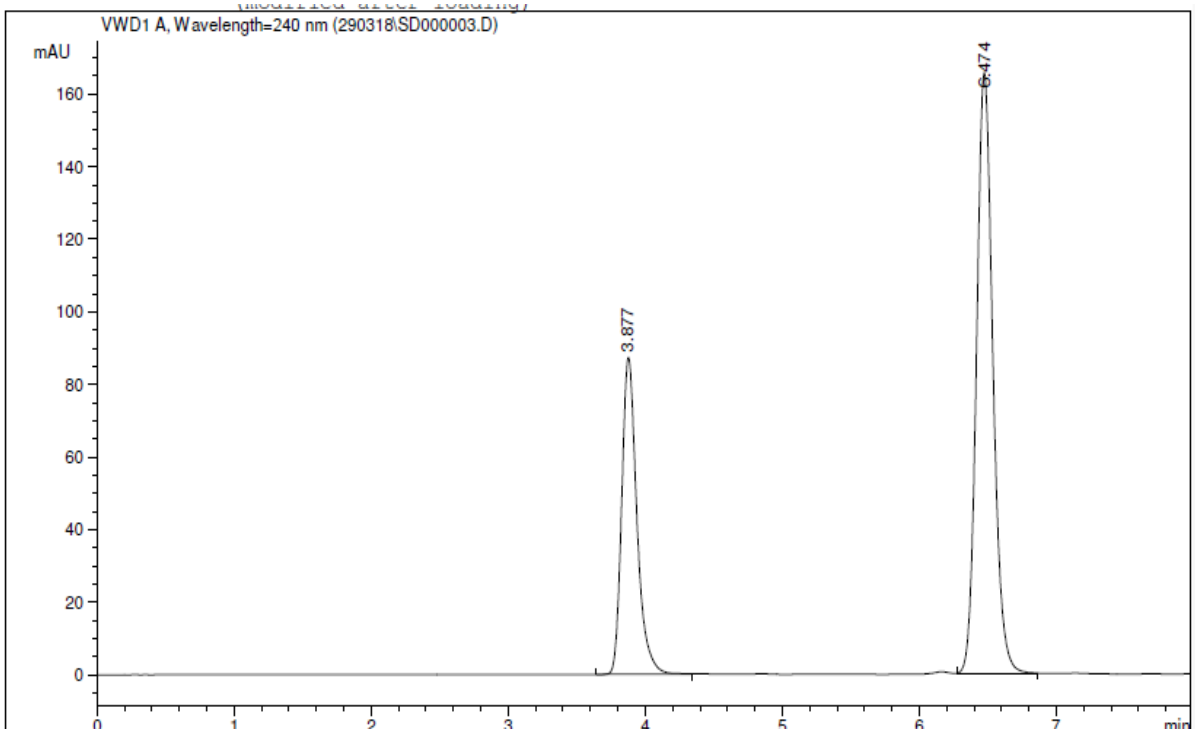


International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





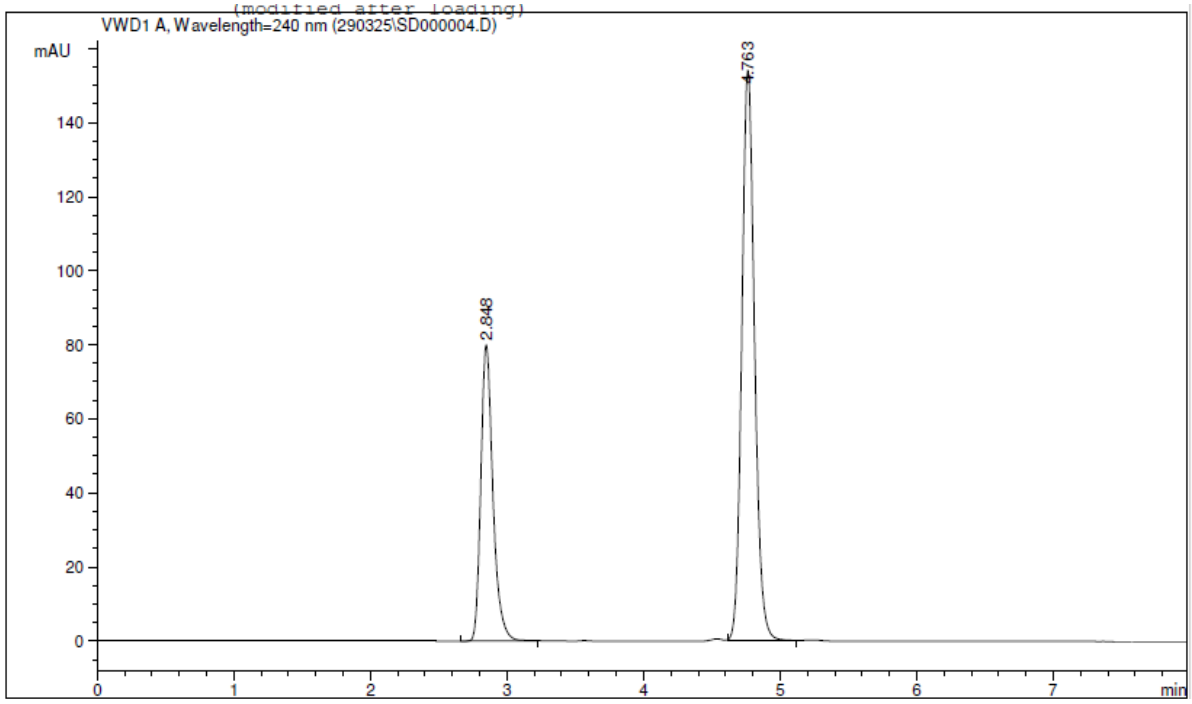
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

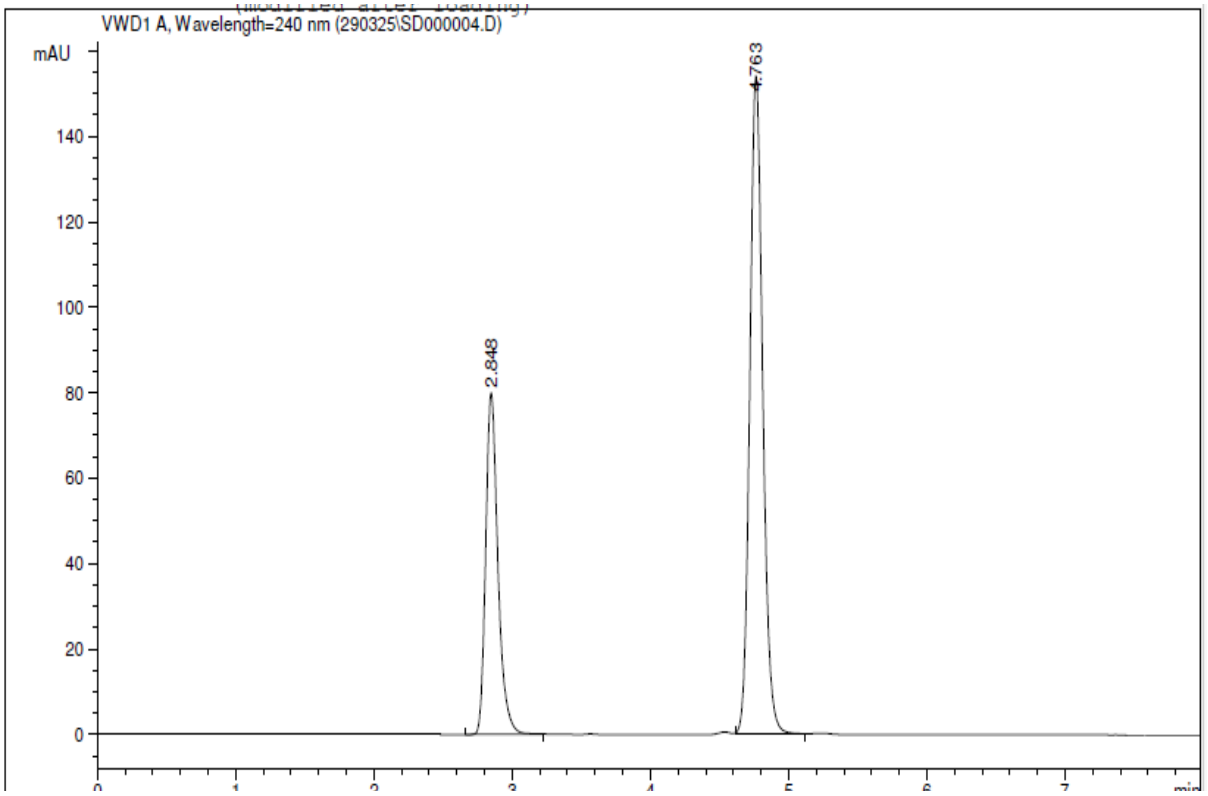


This HPLC chromatogram, detected at 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 2.848 minutes and 4.763 minutes. The peak at 4.763 minutes exhibits a significantly higher detector response (measured in milli-absorbance units, mAU) compared to the peak at 2.848 minutes, suggesting a greater concentration of the compound eluting at the later time under the given chromatographic conditions. (h) This HPLC chromatogram, obtained with detection at 240 nm, illustrates the separation of two chemical compounds, represented by two distinct peaks eluting at retention times of approximately 2.848 minutes and 4.763 minutes. The peak at 4.763 minutes shows a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 2.848 minutes, indicating a greater absorbance or concentration of the compound eluting at the later retention time under these chromatographic conditions. (i) This HPLC chromatogram, detected at 240 nm, displays the separation of two compounds, evident as two distinct peaks eluting at retention times of approximately 3.348 minutes and 5.628 minutes. The peak at 5.628 minutes exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.348 minutes, indicating a greater concentration of the compound eluting at the later retention time in the analyzed sample. (j) This HPLC chromatogram, obtained with detection at 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 3.348 minutes and 5.628 minutes. The peak observed at 5.628 minutes exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.348 minutes, suggesting a substantially greater concentration of the compound corresponding to the later elution time in the analyzed sample. (k) This HPLC chromatogram, detected at 240 nm, shows the separation of two compounds, evident as two distinct peaks eluting at retention times of approximately 3.356 minutes and 5.652 minutes. The peak at 5.652 minutes exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.356 minutes, indicating a greater concentration of the compound eluting at the later retention time in the analyzed sample. (l) This HPLC chromatogram, obtained with detection at 240 nm, shows the separation of two compounds, indicated by two distinct peaks eluting at retention times of approximately 3.356 minutes and 5.652 minutes. The peak observed at 5.652 minutes exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the peak at 3.356 minutes, suggesting a substantially greater

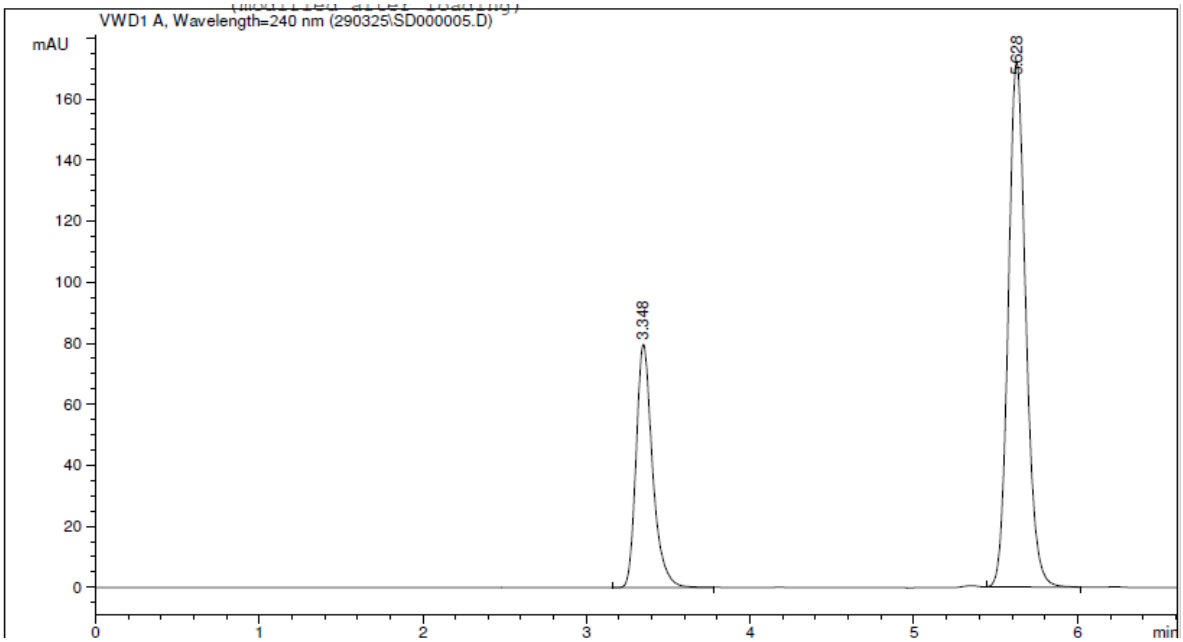
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

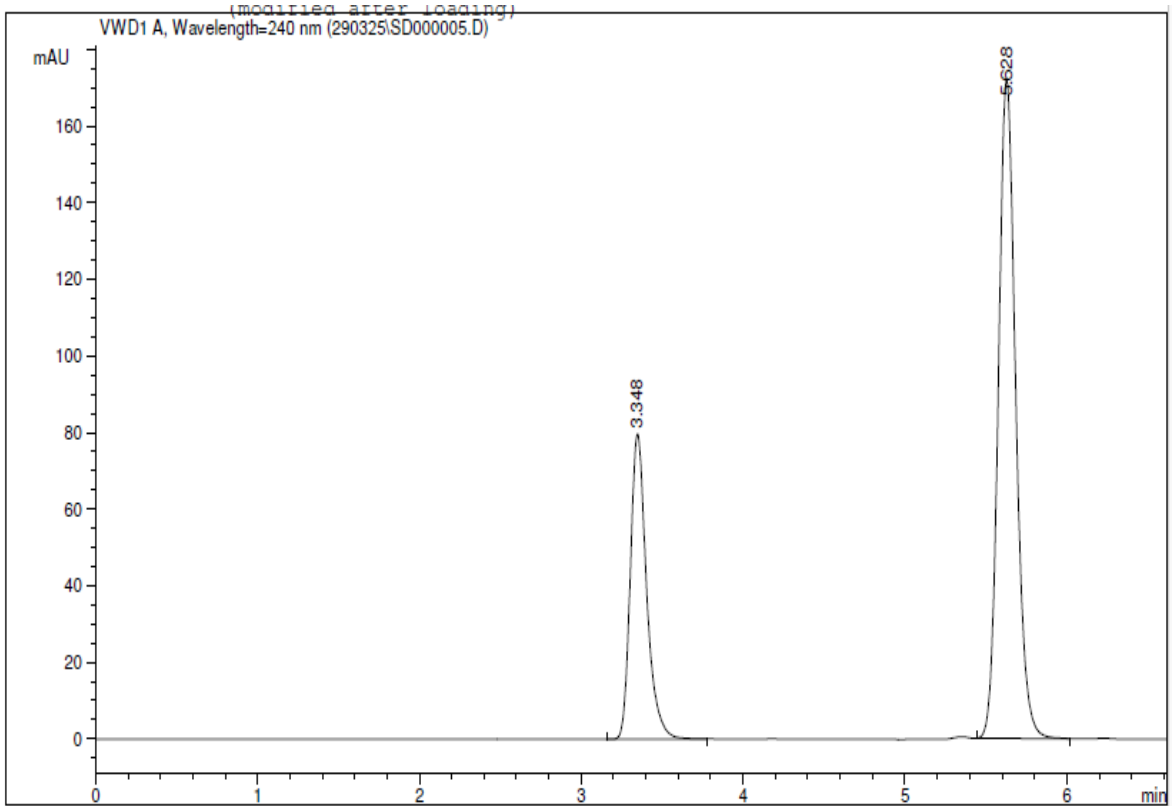
concentration of the compound corresponding to the later elution time in the analyzed sample.





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

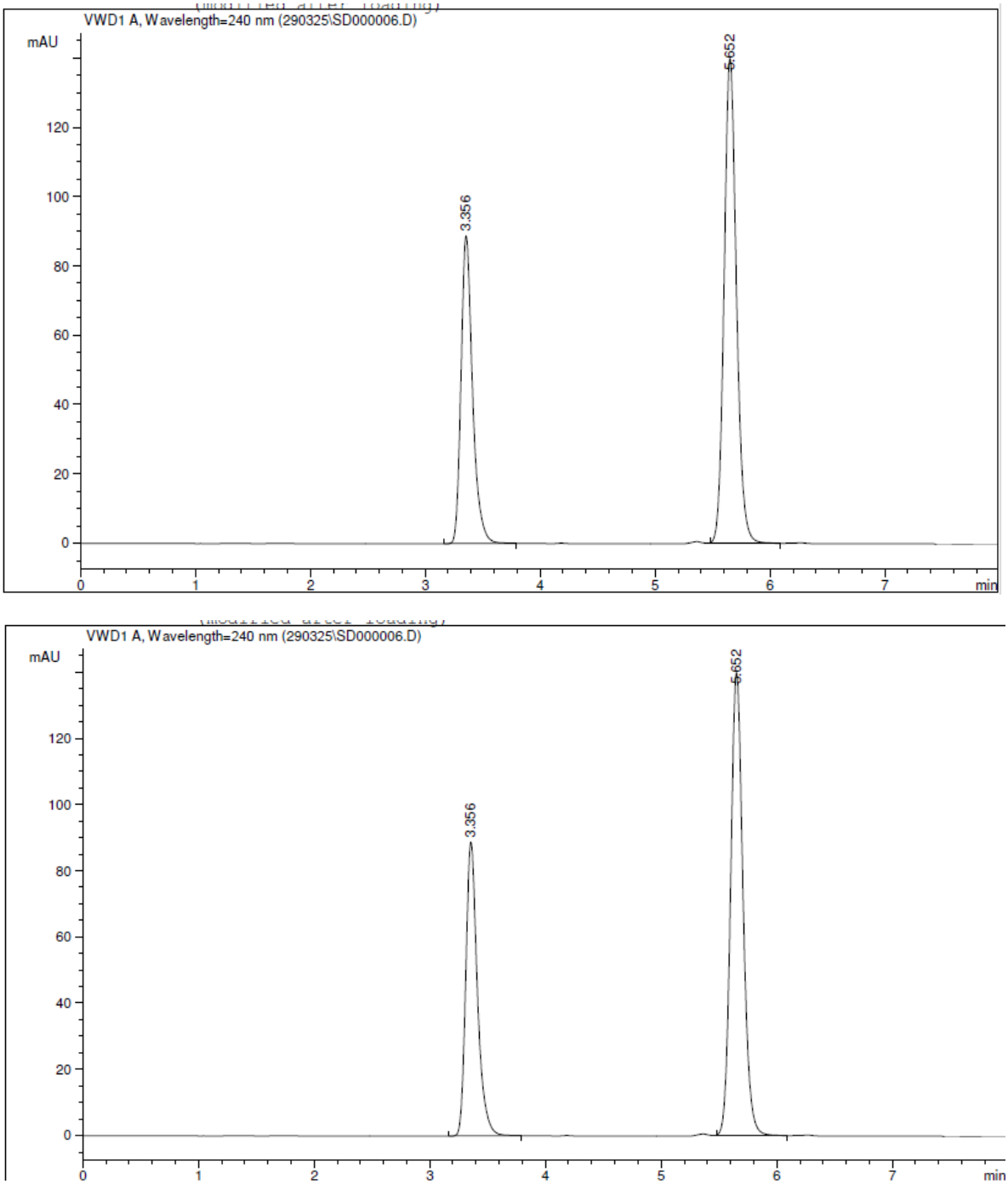


Figure 6: Robustness of two compound Formoterol and Tiotropium

➢ **Rougudness**

This High-Performance Liquid Chromatography (HPLC) chromatogram, with detection at 240 nm, shows the separation of two compounds identified as Formoterol and Tiotropium. Formoterol elutes with a retention time of about 3.342 minutes, whereas Tiotropium elutes later with a retention time of about 5.676 minutes. Peak height and area for Tiotropium are much larger than those of Formoterol, reflecting greater concentration of Tiotropium compared to Formoterol in the sample analyzed. (b) This HPLC chromatogram detected at 240 nm illustrates the separation of Tiotropium and Formoterol.

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

Formoterol elutes with a retention time of about 3.306 minutes, whereas Tiotropium elutes at a later retention time of about 5.595 minutes. The peak height and area for Tiotropium are significantly greater than those for Formoterol, indicating a higher concentration of Tiotropium relative to Formoterol in the analyzed sample.Such reproducibility across different analysts and instruments confirms the method's ruggedness. Similar conclusions were reached by Subrahmanyam et al., who emphasized the importance of ruggedness for regulatory compliance in pharmaceutical manufacturing [8].

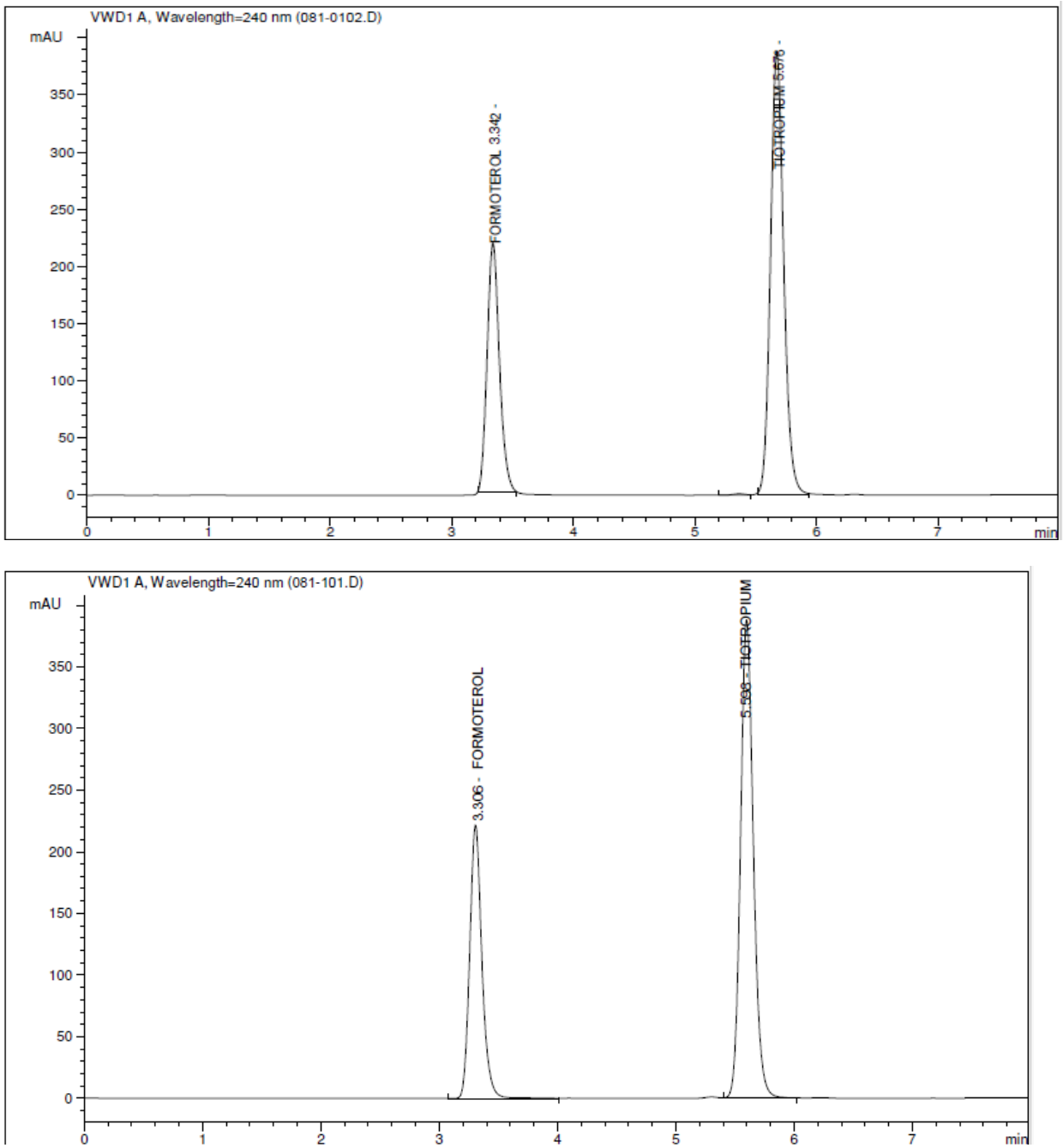
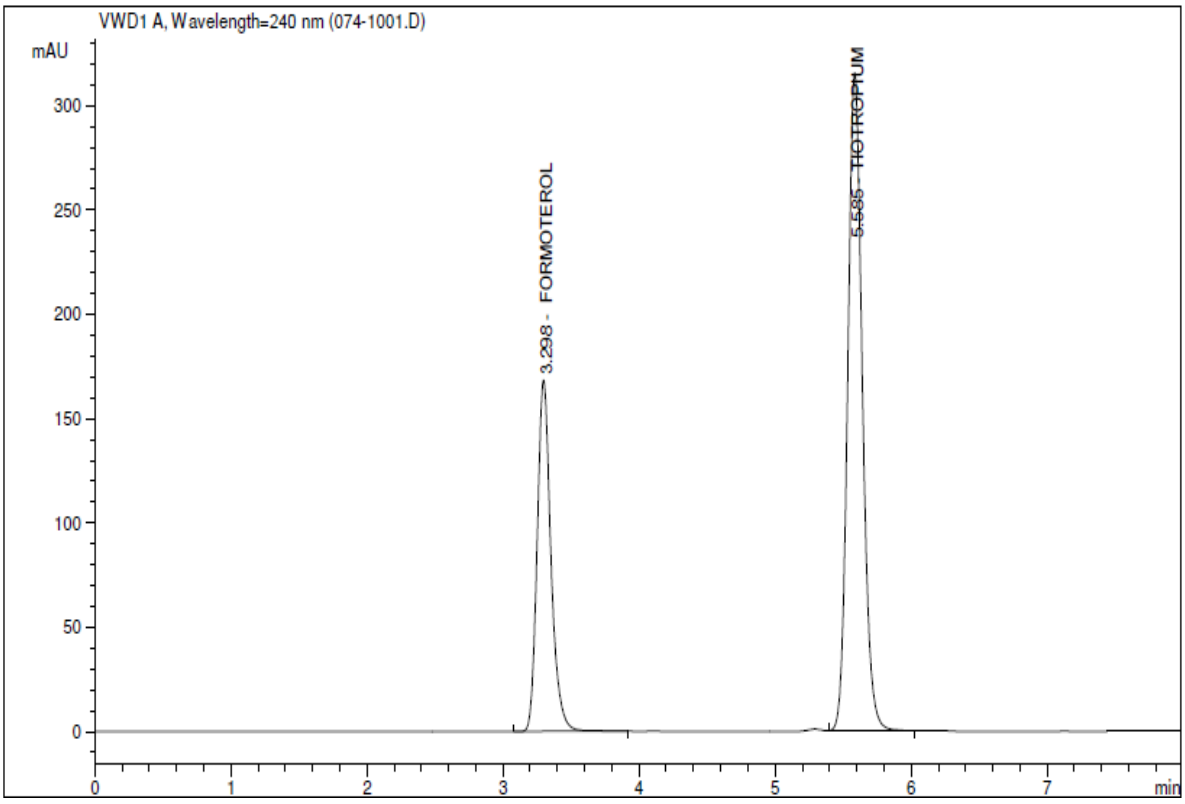


Figure 7: shows the Rougudness of separation of two compounds identified as Formoterol and Ipratropium

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

➢ **Assay**

This HPLC chromatogram, obtained with UV detection at 240 nm, shows the separation of two compounds, identified as Formoterol and Tiotropium. Formoterol elutes at a retention time of approximately 3.298 minutes, while Tiotropium elutes later at approximately 5.585 minutes. The peak corresponding to Tiotropium exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the Formoterol peak, indicating a higher concentration of Tiotropium relative to Formoterol in the analyzed sample. (b) This HPLC chromatogram, detected at 240 nm, shows the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of approximately 3.275 minutes, while Tiotropium elutes later at approximately 5.532 minutes. The peak height and area for Tiotropium are significantly greater than those for Formoterol, indicating a higher concentration of Tiotropium relative to Formoterol in the analyzed sample.Patil and Rane reported similar outcomes when applying validated HPLC methods to capsule formulations containing the same combination [9]. These findings confirm that the developed method is accurate, suitable, and reliable for routine pharmaceutical analysis.



International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

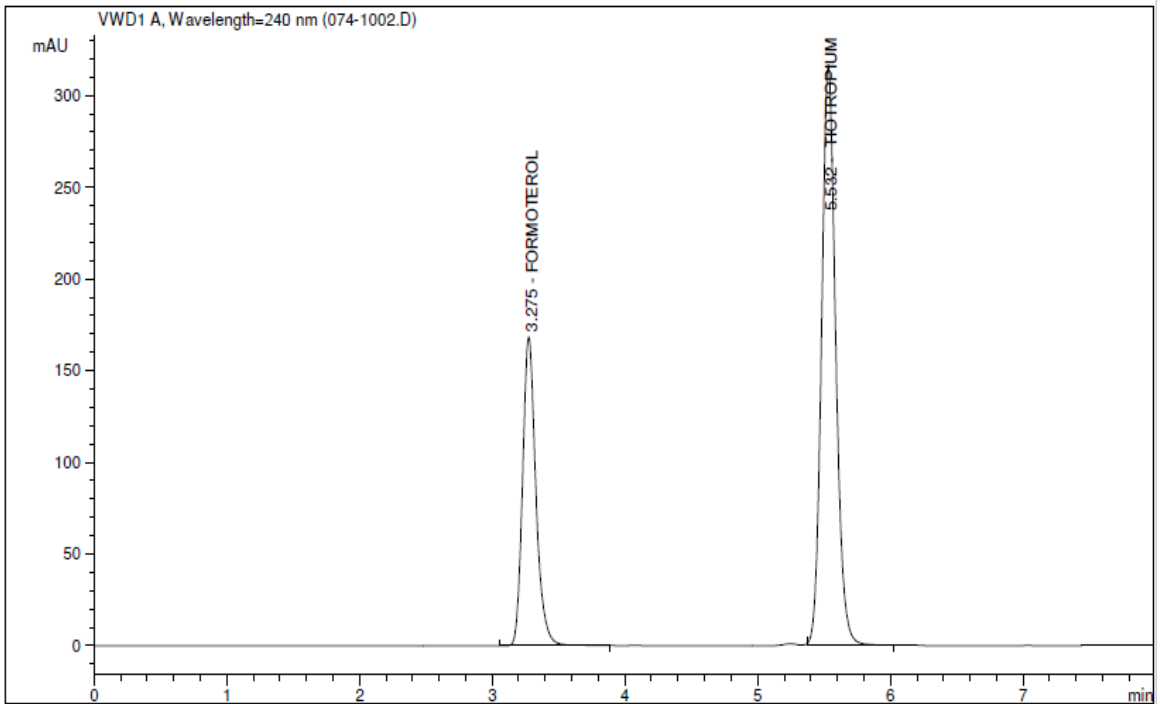


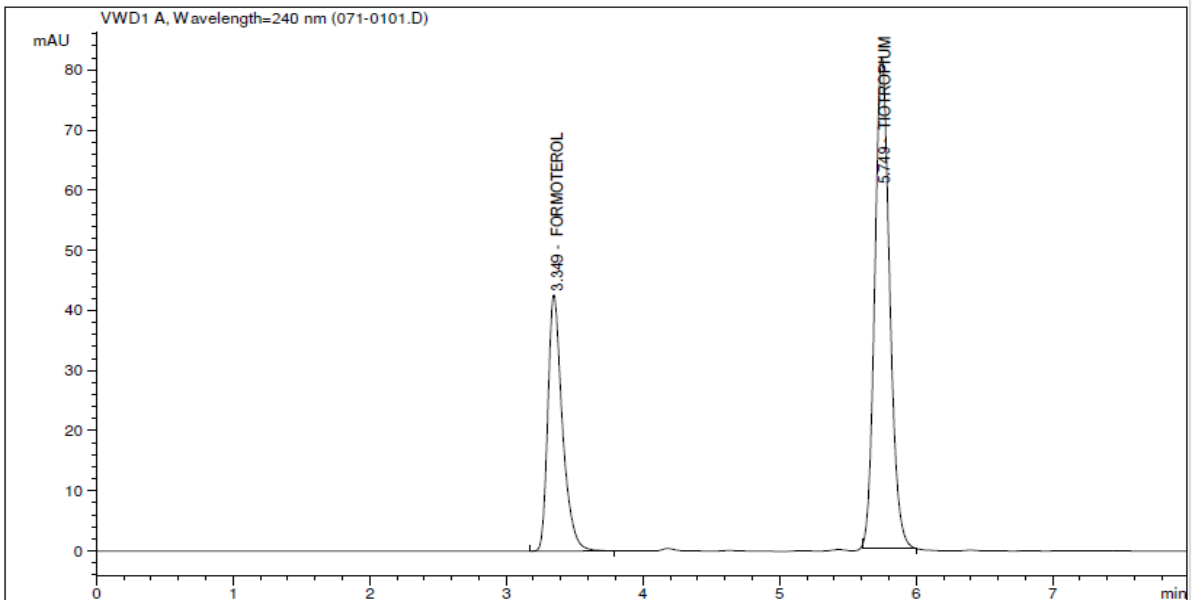
Figure 8: assay of of two compounds, identified as Formoterol and Ipratropium

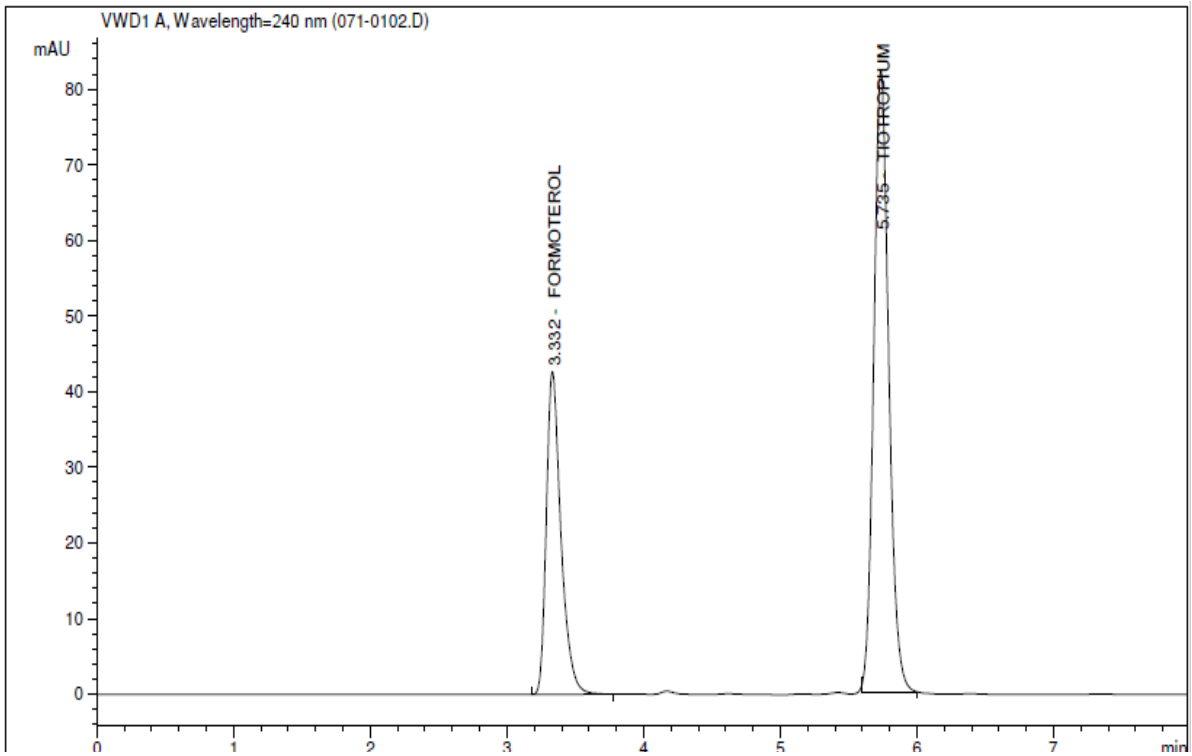
➢ **Linearity**

This HPLC chromatogram, obtained with UV detection at 240 nm, shows the separation of two compounds, identified as Formoterol and Tiotropium. Formoterol elutes at a retention time of approximately 3.349 minutes, while Tiotropium elutes later at approximately 5.749 minutes. The peak corresponding to Tiotropium exhibits a significantly higher intensity (measured in milli-absorbance units, mAU) compared to the Formoterol peak, indicating a higher concentration of Tiotropium relative to Formoterol in the analyzed sample. (b) This HPLC chromatogram, with UV detection at 240 nm, shows the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of approximately 3.332 minutes, while Tiotropium elutes later at approximately 5.735 minutes. The peak corresponding to Tiotropium is significantly larger than the Formoterol peak, indicating a higher concentration of Tiotropium relative to Formoterol in the analyzed sample. (c) This HPLC chromatogram, detected at 240 nm, shows the separation of Formoterol and Tiotropium. Formoterol elutes with a retention time of about 3.359 minutes, whereas Tiotropium elutes at a later time of about 5.731 minutes. The peak area and height for Tiotropium are much larger than for Formoterol, suggesting a larger amount of Tiotropium compared to Formoterol in the sample being analyzed. (d) This HPLC chromatogram, which was obtained using UV detection at 240 nm, illustrates the separation of Tiotropium and Formoterol. Formoterol elutes at about 3.333 minutes of retention time, whereas Tiotropium elutes at about 5.680 minutes. The Tiotropium peak is much larger compared to the Formoterol peak, reflecting a greater concentration of Tiotropium compared to Formoterol in the sample under analysis. (e) This HPLC chromatogram, detected at 240 nm, illustrates the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of around 3.310 minutes, whereas Tiotropium elutes at a later time of around 5.655 minutes. Peak height and area for Tiotropium are significantly larger than those for Formoterol, reflecting an increased concentration of Tiotropium over Formoterol in the sample under analysis. (f) This HPLC chromatogram, with UV detection at 240 nm, demonstrates the resolution of Formoterol and Tiotropium. Formoterol elutes around 3.361 minutes of retention time, whereas Tiotropium elutes at around 5.745 minutes of retention time. The peak area for Tiotropium is much larger than that of Formoterol, reflecting a higher concentration of Tiotropium compared to Formoterol in the sample being analyzed. The results agree with those of Rahman et al., who achieved R^2 values above 0.998 using their stability-indicating RP-HPLC method for

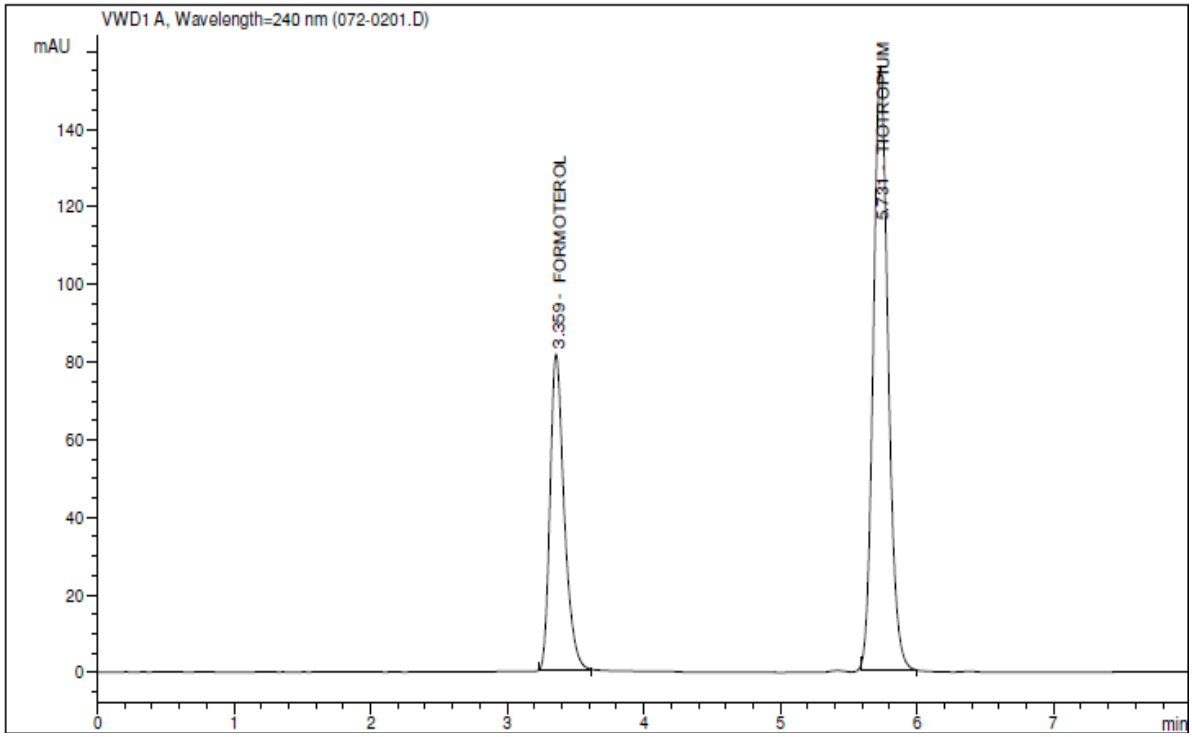
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

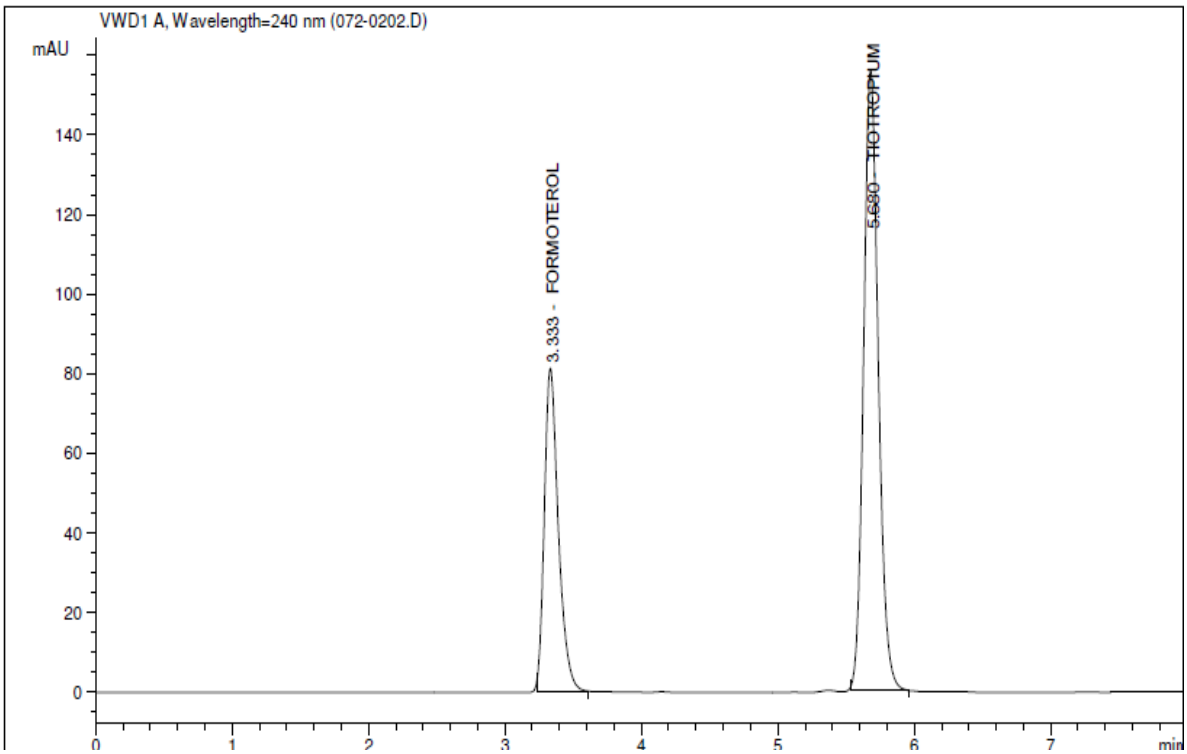
Formoterol [3]. Linearity validation validates the applicability of the method for a broad spectrum of formulations and strength ranges in commercial formulations.



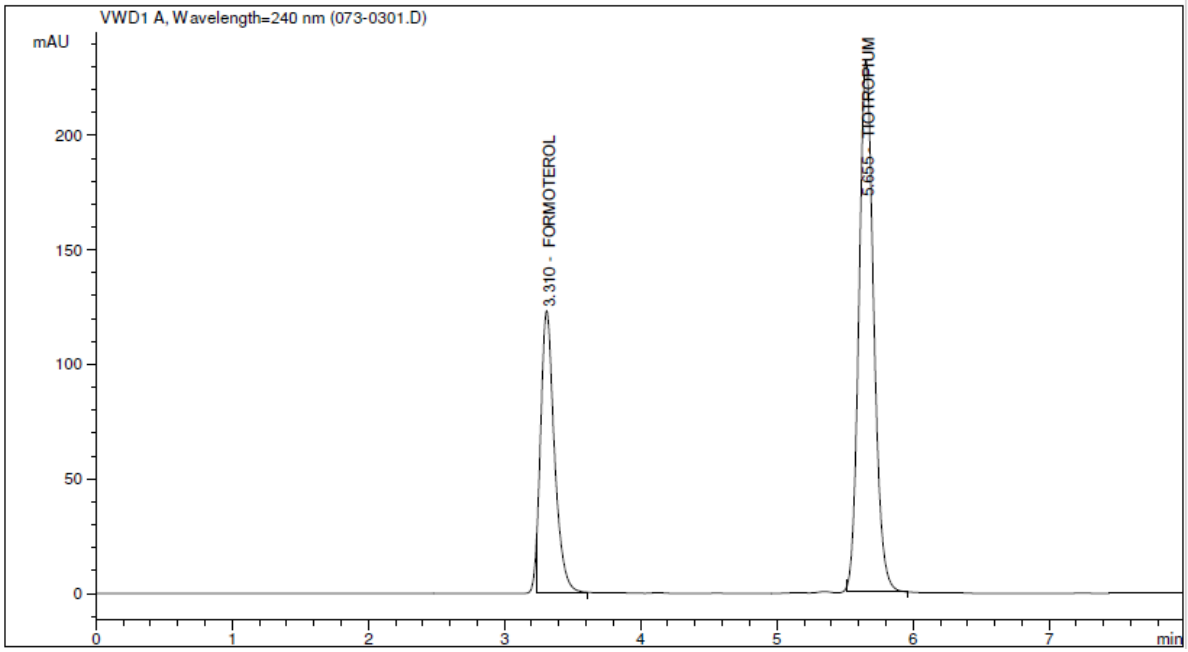


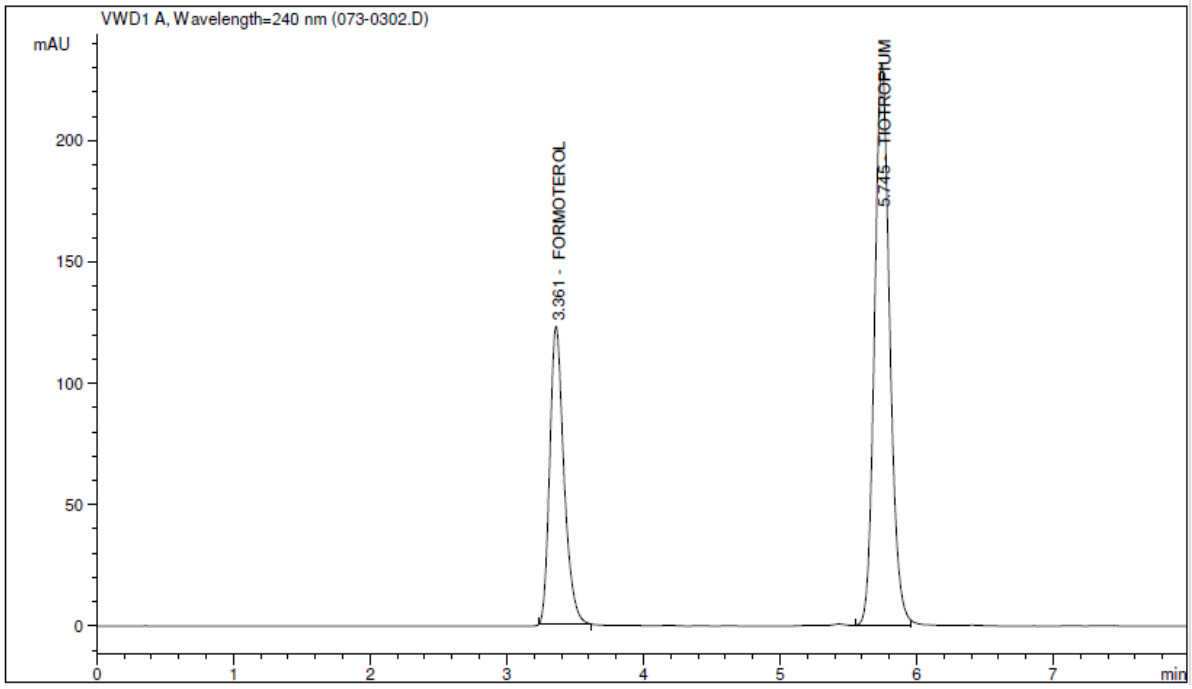
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

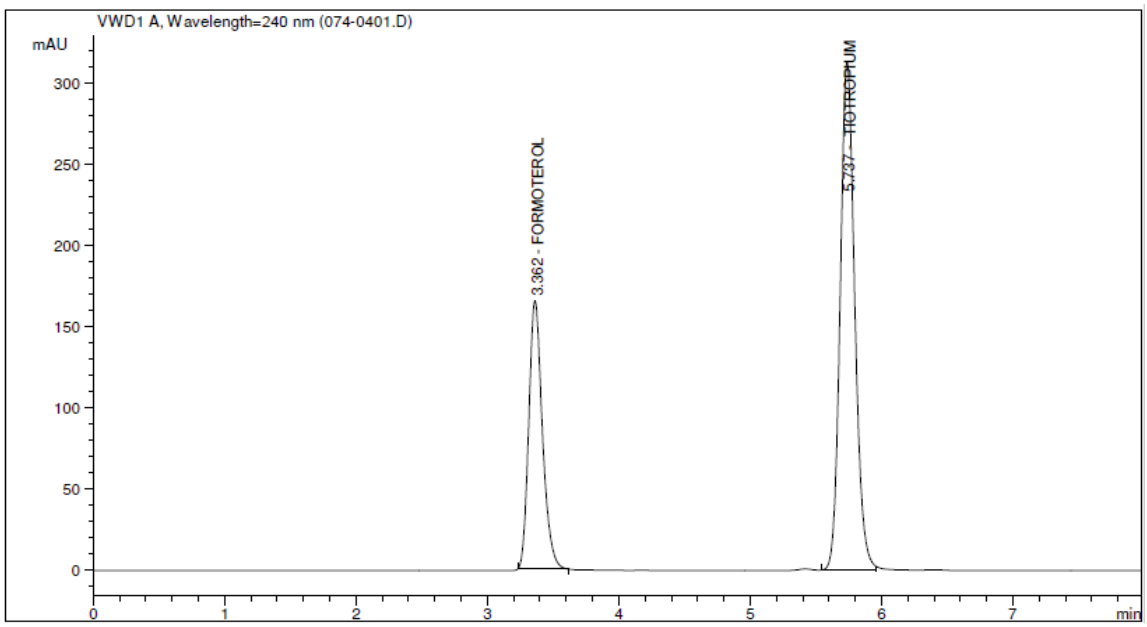


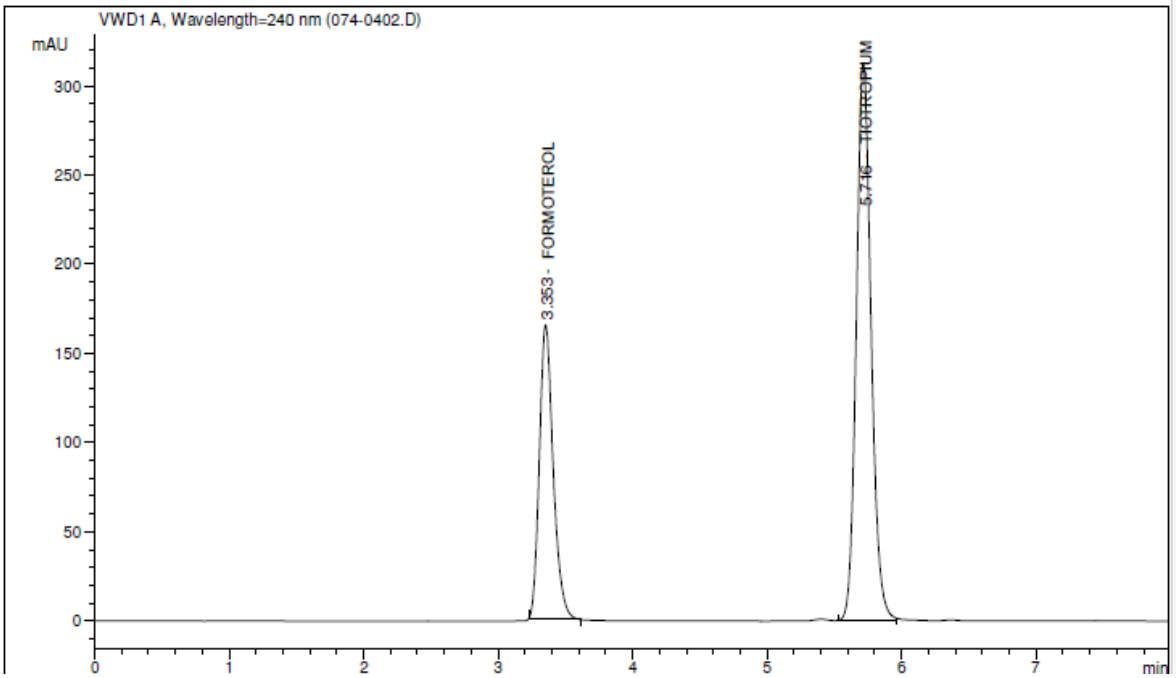


This HPLC chromatogram, with UV detection at 240 nm, indicates the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of about 3.362 minutes, while Tiotropium elutes later at about 5.737 minutes. The Tiotropium peak is much greater than the Formoterol peak, which means that there is a greater concentration of Tiotropium compared to Formoterol in the sample analyzed. (h) This HPLC chromatogram at 240 nm demonstrates the resolution of Formoterol and Tiotropium. Formoterol elutes with a retention time of around 3.353 minutes, whereas Tiotropium elutes at a later retention time of around 5.716 minutes. The area and peak height for Tiotropium are considerably larger than for Formoterol, which signifies that Tiotropium is present in higher concentration compared to Formoterol in the sample analyzed. (i)

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

This HPLC chromatograph, which was recorded using UV detection at 240 nm, illustrates the resolution of Formoterol and Tiotropium. Formoterol emerges at a retention time of about 3.345 minutes, whereas Tiotropium emerges later at about 5.688 minutes. The Tiotropium peak is much larger than the Formoterol peak, suggesting a greater concentration of Tiotropium compared to Formoterol in the sample under analysis. (j) This HPLC chromatogram, which is detected at 240 nm, demonstrates the resolution of Formoterol and Tiotropium. Formoterol elutes with a retention time of around 3.340 minutes, whereas Tiotropium elutes later at around 5.673 minutes. The area and height of the peak of Tiotropium are considerably higher than that of Formoterol, signifying a higher concentration of Tiotropium compared to Formoterol in the sample analyzed.





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

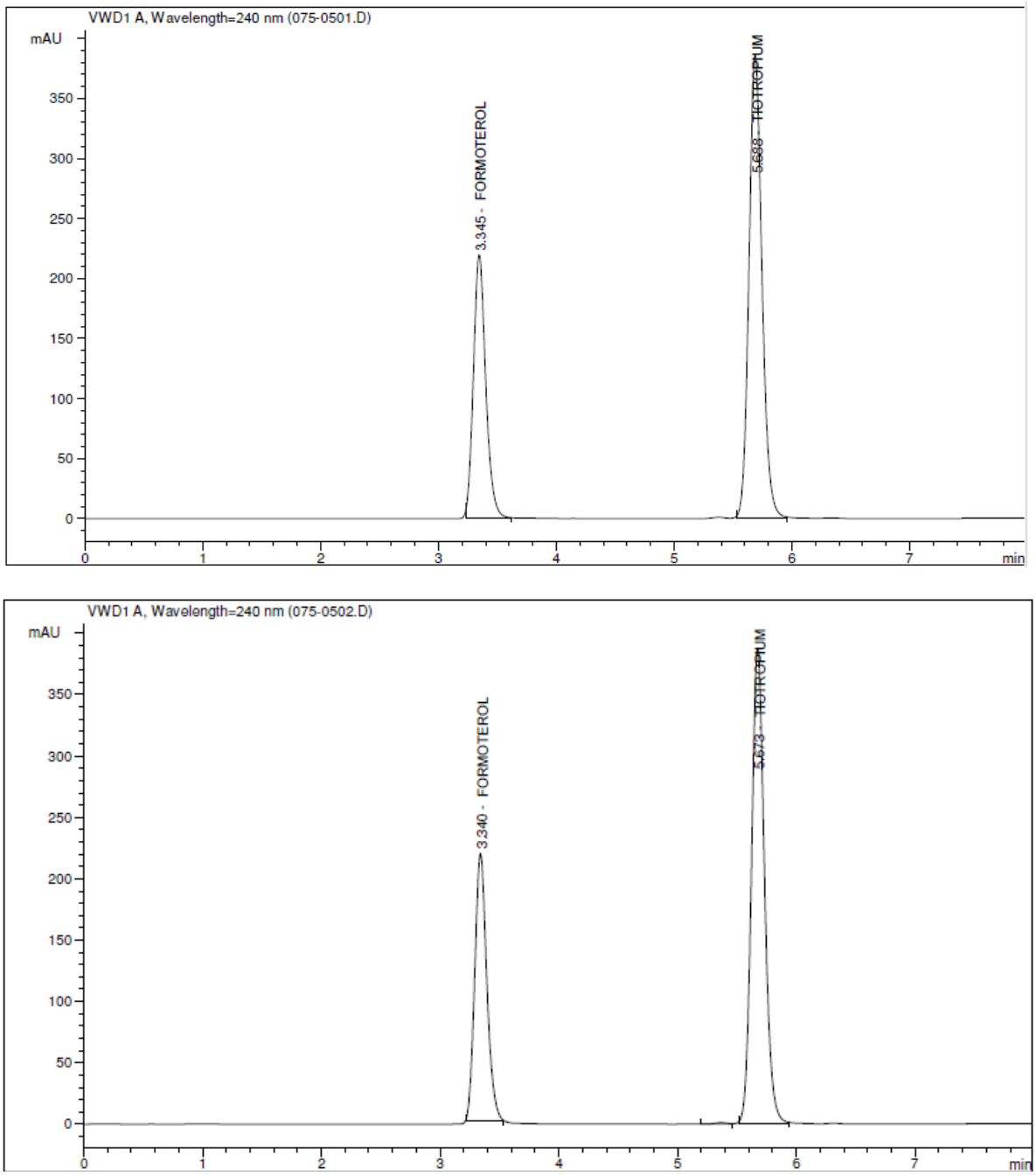


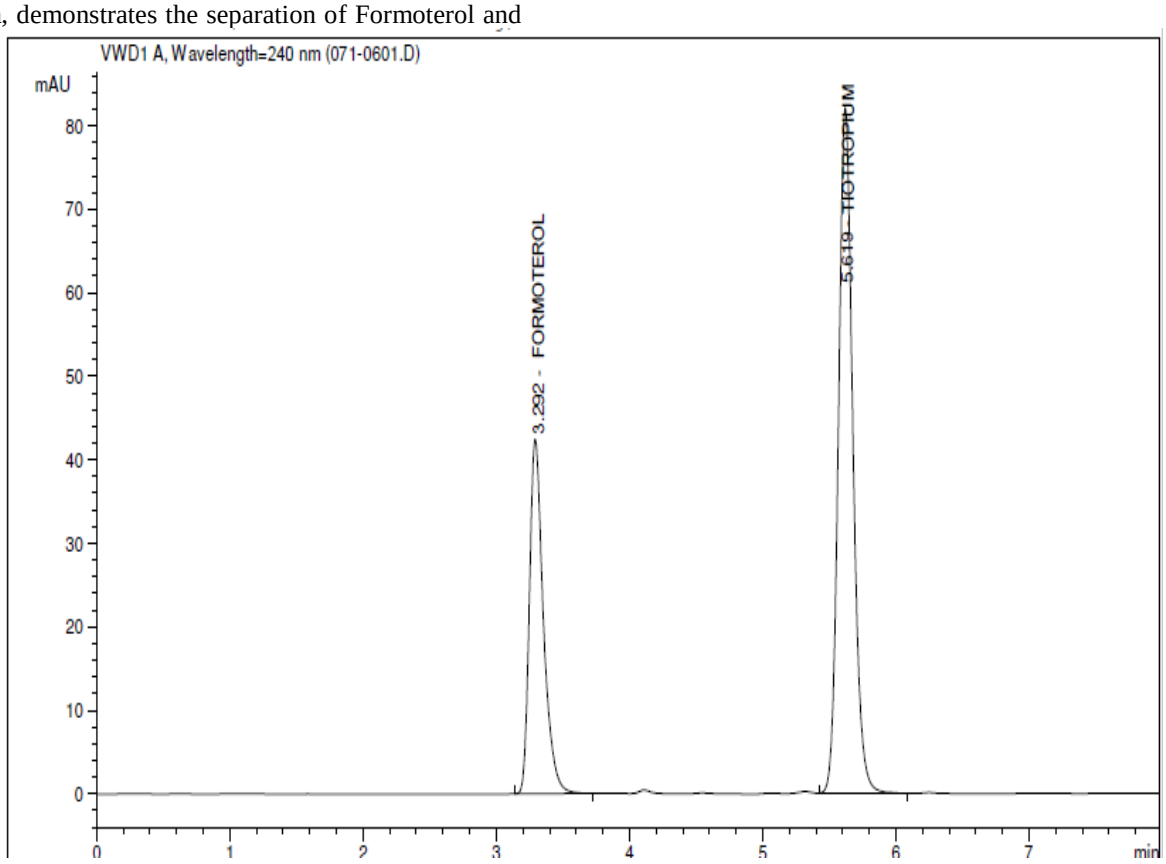
Figure 9: This HPLC chromatogram, obtained with UV detection at 240 nm, shows the separation of two compounds, identified as Formoterol and Tiotropium

➢ **Precesion**

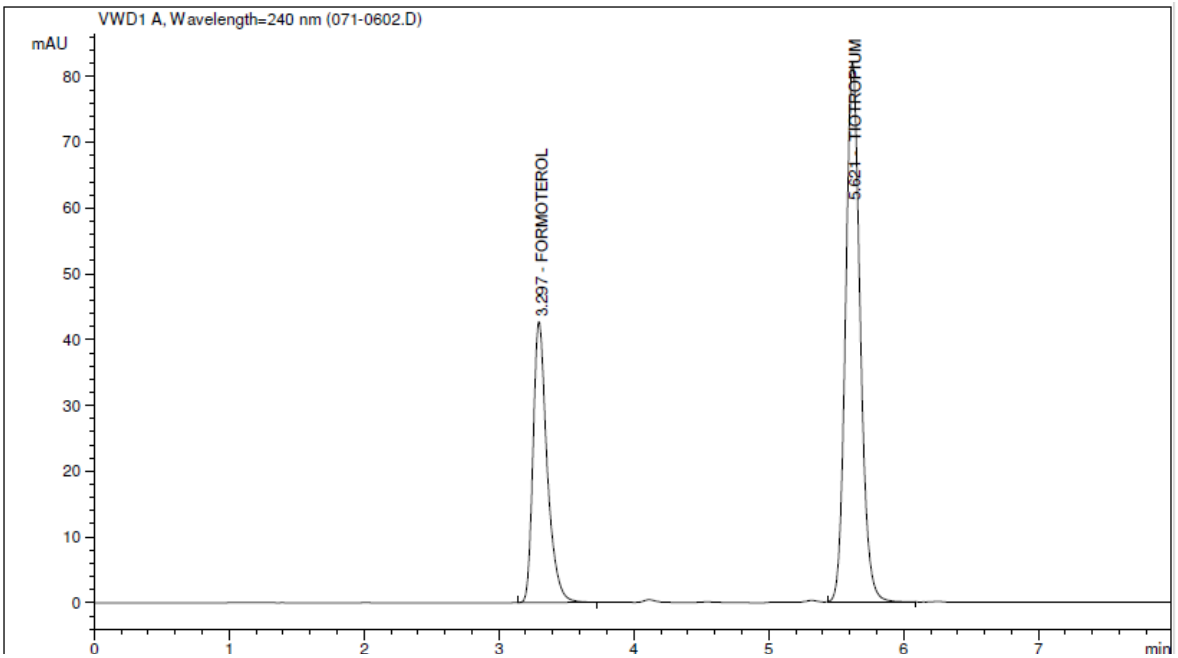
This HPLC chromatograph, which is run using UV detection at 240 nm, indicates the resolution of Formoterol and Tiotropium. Formoterol elutes around a retention time of around 3.292 minutes, and Tiotropium elutes later at around 5.619 minutes. The peak of Tiotropium is considerably larger compared to the peak of Formoterol, indicating that there was a greater amount of Tiotropium compared to Formoterol in the sample that has been analyzed. (b) This HPLC chromatogram, which was detected at 240 nm, indicates the separation of Tiotropium and Formoterol. Formoterol elutes at around 3.297 minutes, while Tiotropium elutes at around 5.621 minutes. The height and area of the peak for

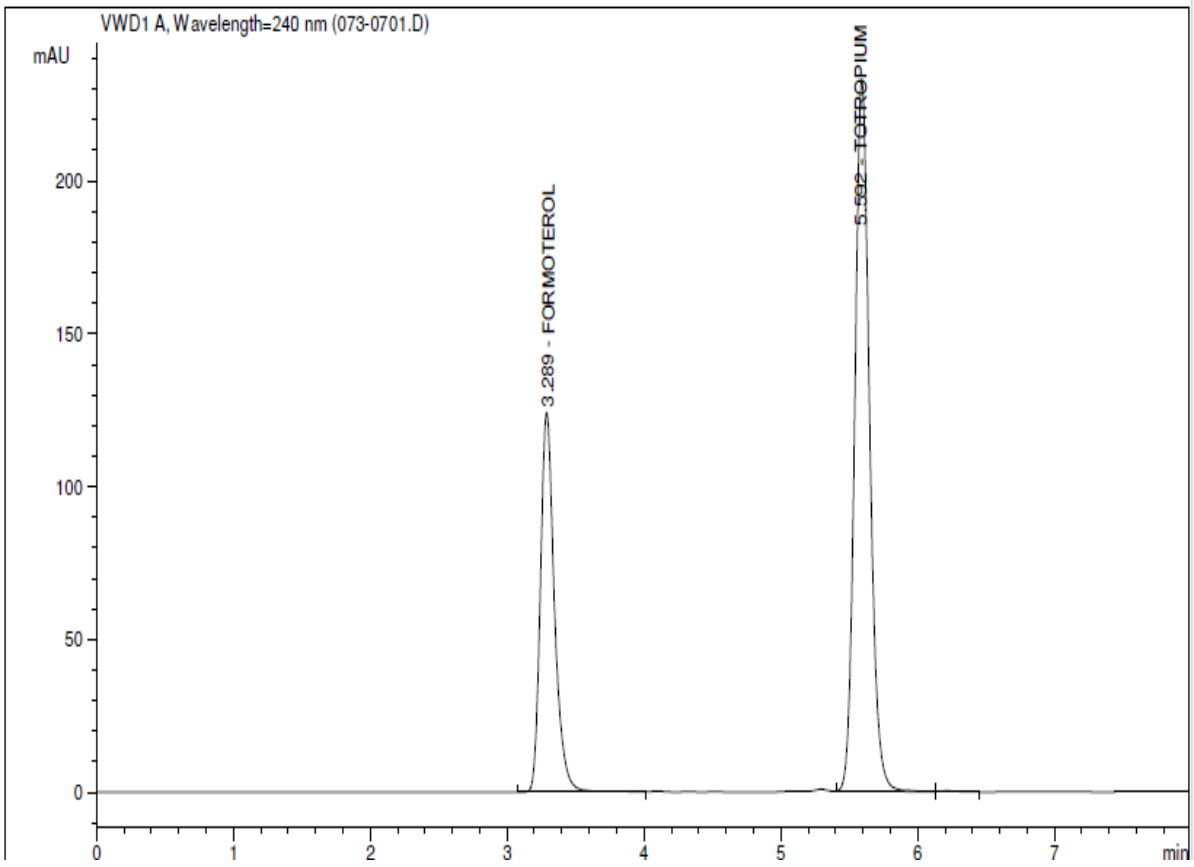
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

Tiotropium are significantly higher than those of Formoterol, reflective of a greater concentration of Tiotropium compared to Formoterol in the sample that was analyzed. (c) This HPLC chromatogram, with UV detection at 240 nm, demonstrates the separation of Formoterol and Tiotropium. Formoterol elutes with a retention time of about 3.289 minutes, whereas Tiotropium elutes at a later retention time of about 5.592 minutes. The Tiotropium peak is much larger compared to the Formoterol peak, showing a greater concentration of Tiotropium compared to Formoterol in the sample that was analyzed. (d) This HPLC chromatogram, which was detected at 240 nm, indicates the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of about 3.298 minutes, whereas Tiotropium elutes later at about 5.596 minutes. The peak area and height of Tiotropium are significantly higher than those of Formoterol, reflecting a greater concentration of Tiotropium compared to Formoterol in the sample analyzed. (e) This HPLC chromatogram, recorded using UV detection at 240 nm, demonstrates the separation of Formoterol and Tiotropium. Formoterol elutes at a retention time of around 3.314 minutes, whereas Tiotropium elutes later at around 5.608 minutes. The peak for Tiotropium is much larger compared to the Formoterol peak, reflecting a greater concentration of Tiotropium compared to Formoterol in the sample analyzed. (f) This HPLC chromatogram, monitored at 240 nm, illustrates the separation of Formoterol and Tiotropium. Formoterol elutes with a retention time of about 3.306 minutes, while Tiotropium elutes later with a retention time of about 5.598 minutes. The area and peak height of Tiotropium are much larger than those of Formoterol, reflecting a higher concentration of Tiotropium compared to Formoterol in the sample being analyzed. Kumar and Rao reported similar results, wherein the values of RSD were much less than 2%, stressing that the procedure could be used with confidence for routine determination of respiratory drugs in quality control laboratories [5]. Precision assures the ruggedness of the method under routine laboratory conditions.

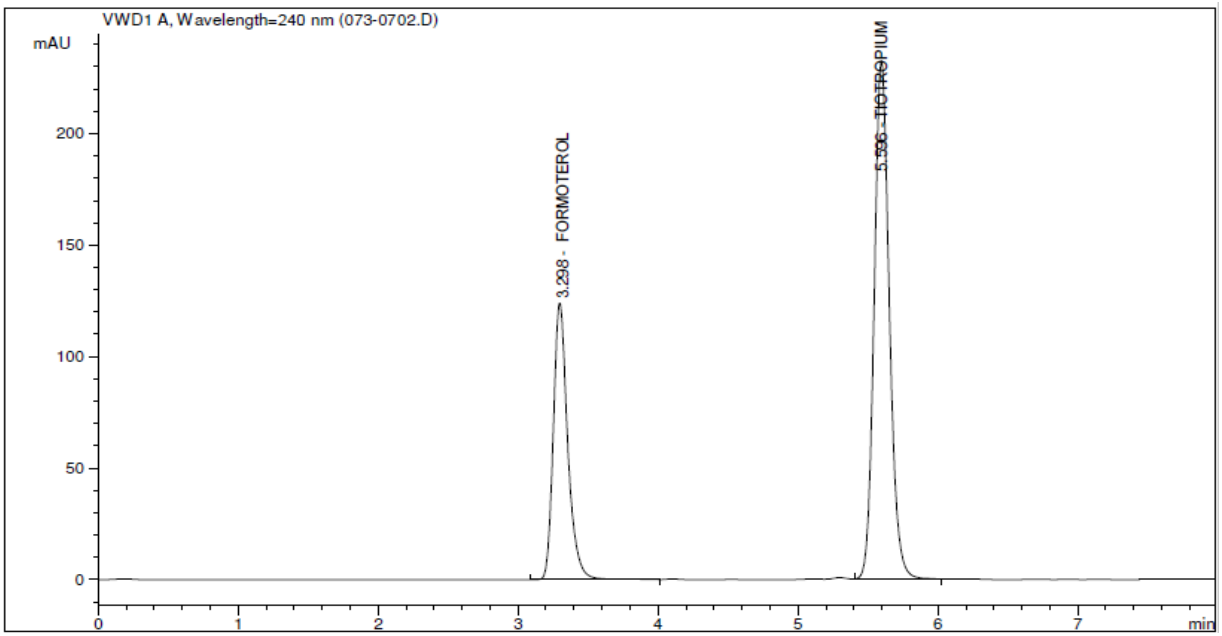


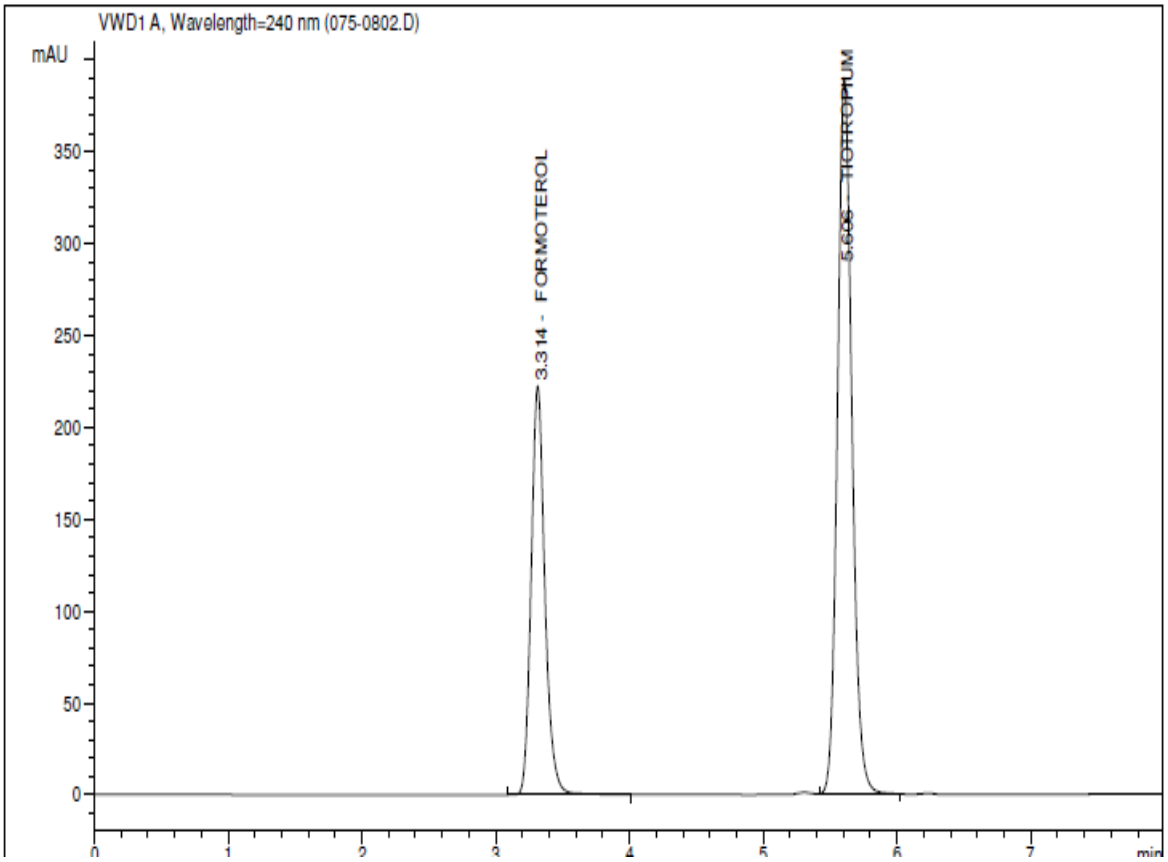
International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494





International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1132-1161 www.ijprajournal.com ISSN: 2456-4494

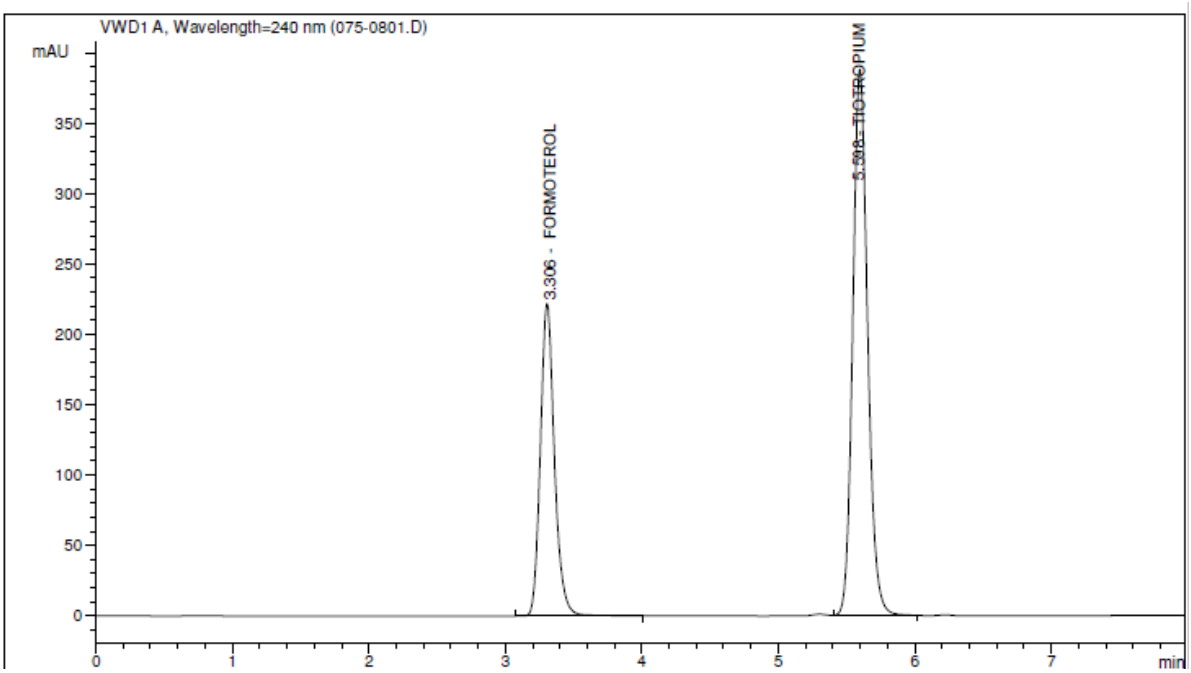


Figure 10: This HPLC chromatograph, which is run using UV detection at 240 nm, indicates the resolution of Formoterol and Tiotropium

IV. CONCLUSION

The optimized HPLC technique efficiently attained the concomitant separation and estimation of Formoterol and Tiotropium in fixed-dose combinations with excellent specificity, precision, and accuracy. Reproducible retention times of about 3.3 minutes for Formoterol and 5.7 minutes for Tiotropium were obtained in replicates, demonstrating outstanding method reproducibility. UV-Vis spectroscopy identified the best detection at 240 nm, which is the isobestic point for both the drugs. Linearity was good (R^2 = 0.9995 for Formoterol), and the robustness tests confirmed the reproducibility of the method under slight intentional changes. The system well discriminated analytes even in complicated mixtures. This method hence presents a validated, reproducible, and effective procedure for routine batch analysis and quality control of bronchodilator combinations in drug preparations.

REFERENCES

[1]. Ni H, Moe S, Soe Z, Myint KT, Viswanathan KN. Combined aclidinium bromide and long-acting beta2-agonist for chronic obstructive pulmonary disease (COPD). Cochrane Database of Systematic Reviews. 2018(12).

[2]. Miravitlles M, Baek S, Vithlani V, Lad R. Optimal bronchodilation for COPD patients: are all long-acting β2-agonist/long-acting muscarinic antagonists the same?. Tuberculosis and respiratory diseases. 2018 Jun 19;81(3):198.

[3]. Blais CM. BRONCHOPROTECTION PROVIDED BY LONG-ACTING MUSCARINIC ANTAGONISTS AND ULTRA LONG-ACTING β2 AGONISTS AGAINST METHACHOLINE-INDUCED BRONCHOCONSTRICTION IN MILD ASTHMATICS (Doctoral dissertation, University of Saskatchewan).

[4]. Kachave RN, Chaudhari SR, Kale M, Wagh RD. Simultaneous estimation of Formoterol fumarate and Tiotropium bromide by RP-HPLC in dry powder inhaler. Inventi Rapid: Pharm Analysis & Quality Assurance. 2013 Aug 3;4:1-4.

[5]. LAKSHMI AJ, ABHISHEK K, PRIYANKA YL, BALAJI P, ASHRITHA N. DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ACLIDINIUM BROMIDE AND FORMOTEROL FUMARATE IN BULK AND INHALER FORMULATION. Int J Curr Pharm Res. 2022;14(5):13-9.

[6]. Gousuddin M. Development of validated analytical methods for the estimation of

Anti-Asthmatic drug, Formoterol Fumarate in Pharmaceutical Dosage forms (Master's thesis, Rajiv Gandhi University of Health Sciences (India)).

[7]. Seckin S, Saglik Aslan S. Simultaneous UPLC Assay for Oxitropium Bromide and Formoterol Fumarate Dihydrate in Pressurized Metered Dose Inhaler Products for Chronic Obstructive Pulmonary Disease. Journal of AOAC International. 2024 Mar 1;107(2):242-7.

[8]. Harps LC, Bizjak DA, Girreser U, Zügel M, Steinacker JM, Diel P, Parr MK. Quantitation of Formoterol, Salbutamol, and Salbutamol-4′-O-Sulfate in Human Urine and Serum via UHPLC-MS/MS. Separations. 2023 Jun 22;10(7):368.

[9]. Prasad K. Spectrophotometric and HPLC Method Development of Simultaneous Estimation of Anti-Tubercular Drugs (Master's thesis, Rajiv Gandhi University of Health Sciences (India)).

[10]. Banoth RK, Nethikoppula RL. A Study of Analytical Method Development, Validation and Forced Degradation for Simultaneous Estimation of Glycopyrrolate and Formoterol Fumarate Bulk Drug by UHPLC Method. International Journal of Pharmaceutical Sciences and Research. 2021;12:3781-90.

[11]. Sule S, Ambadekar S, Singh A, Naik P. A rapid and stability indicating RP-HPLC method for simultaneous determination of tiotropium, formoterol and ciclesonide in a dry powder inhaler. World J Pharm Res. 2014 Apr 5;3:819-30.

[12]. Gondhale PS, Cheriyan BV. Quantitative determination of related substances in formoterol fumarate and tiotropium in tiomatetranscaps® dry powder inhaler. Turkish Journal of Pharmaceutical Sciences. 2022 Feb 28;19(1):35.

[13]. Zanwar AS, Sen DB, Sen AK, Seth AK. Simultaneous estimation of mometasone furoate and formoterol fumarate by HPLC method in rotacaps. Int J Pharm Pharm Sci. 2019 Feb 1;11(2):12-6.

[14]. Parmar VK, Patel HN, Patel BK. Sensitive and robust methods for simultaneous determination of beclomethasone dipropionate and formoterol fumarate dihydrate in rotacaps. Journal of chromatographic science. 2014 Nov 1;52(10):1255-66.

[15]. Elkady EF, Tammam MH, Elmaaty AA. Development and validation of RP-HPLC method for simultaneous estimation of tiotropium bromide, formoterol fumarate, and olodaterol HCL in bulk and metered dose aerosols: Application to olodaterol HCL forced degradation study and degradation kinetics. Chromatographia. 2017 Dec;80:1749-60.

[16]. Attimarad M, Alnajjar A, Sreeharsha N, Nair AB. Development and validation of a RP-HPLC method for simultaneous determination of formoterol fumarate and tiotropium bromide. J Chromatogr Sci. 2014;52(9):901–906.

[17]. Reddy M, Bhargavi K, Rao B. Development and validation of RP-HPLC method for simultaneous estimation of formoterol fumarate and tiotropium bromide. Der Pharmacia Lettre. 2015;7(3):134–142.

[18]. Shabir GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US FDA, USP and ICH. J Chromatogr A. 2003;987(1–2):57–66.

[19]. Desai A, Patel H. RP-HPLC method development and validation for simultaneous estimation of formoterol fumarate and tiotropium bromide in their combined dosage form. Int J Pharm Sci. 2013;5(4):554–558.

[20]. Patel N, Shah D, Shah V. Development and validation of RP-HPLC method for simultaneous estimation of formoterol fumarate and tiotropium bromide in combined dosage forms. Int J Pharm Pharm Sci. 2012;4(5):309–313.

[21]. Joshi SJ, Patel NJ. Simultaneous determination of formoterol fumarate and tiotropium bromide in dry powder inhaler by RP-HPLC. J Pharm Anal. 2012;2(6):460–465.

[22]. Subrahmanyam EVS, Reddy VK, Kumar P. A validated RP-HPLC method for the estimation of formoterol fumarate and tiotropium bromide in combined dosage forms. Int J Pharm Sci Res. 2012;3(5):1347–1352.

[23]. Patil S, Rane V. Development and validation of RP-HPLC method for

simultaneous estimation of formoterol fumarate and tiotropium bromide in inhalation capsules. Indian Drugs. 2011;48(1):36–39.

[24]. Rahman N, Azmi SNH. Simultaneous determination of formoterol and budesonide using validated stability-indicating RP-HPLC method. J Pharm Biomed Anal. 2007;43(2):586–593.

[25]. Kumar BV, Rao ME. Development and validation of stability-indicating RP-HPLC method for simultaneous estimation of tiotropium bromide and formoterol fumarate. Int J PharmTech Res. 2010;2(1):34–42.