

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING REVERSE PHASE HIGH PERFORMACE LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF UMECLIDINIUM AND VILANTEROL IN PHARMACEUTICAL DOSAGE FORM

ABSTRACT

A Sensitive, fast, linear and accurate liquid chromatography technique was developed for the simultaneous determination of Umeclidinium and Vilanterol in Powder dosage form. The estimation was carried out using Phenomenex C₁₈ column (150 × 4.6 mm, 5μ) with ammonium acetate: acetonitrile taken in the ratio 60:40 as mobile phase and pumped at a flow rate of 0.9 ml/min at 30°C. Detection wavelength selected was 245 nm. Retention times of Umeclidinium and Vilanterol were found to be 2.219 min and 2.794 min. The method was validated in terms of linearity, precision, accuracy, limit of detection, limit of quantification as per International council for harmonization guidelines. Degradation studies performed indicated the stability of the drug. All of these analytical validation parameters were evaluated, and the percent relative standard deviations were calculated, indicating the method's suitability for determination of Umeclidinium and Vilanterol in pharmaceutical dosage form.

Keywords: Umeclidinium, Vilanterol, RP – HPLC, Stability – indicating, Anoro Ellipta.

1. INTRODUCTION

Vilanterol (VI), chemically, 4-[(1R)-2-[(6-{2-[(2, 6-dichlorophenyl) methoxy] ethoxy} hexyl) amino]-1-hydroxyethyl]-2-(hydroxymethyl) phenol (Fig.1 (A)) is a long-acting, selective beta₂-adrenergic agonist (LABA) with intrinsic 24-hour action for once daily COPD and asthma diagnosis. The activation of intracellular adenylyl cyclase, which catalyses the conversion of adenosine triphosphate (ATP) to cyclic-3', 5'-adenosine monophosphate, is responsible for its pharmacological action (cAMP). Increases in cyclic AMP are linked to relaxation of the bronchial smooth muscle and suppression of

the release of hypersensitivity mediators from mast cells in the lungs [1].

Umeclidinium (UMEC) (Fig.1 (B)) is a long-acting muscarinic antagonist (LAMA), used to treat COPD symptoms as a preventive treatment. Chemically, it is 1-[2-(benzyloxy) ethyl]-4-(hydroxydiphenylmethyl)-1-azabicyclo [2-2-2] octan-1-ium bromide. It comes as a single-dose inhalation monotherapy or as a combination of a fixed-dose medication with VI, a long-acting beta2-agonist [2]. COPD is a chronic obstructive pulmonary disease characterised by shortness of breath, cough, sputum production, and persistently reduced airflow (less than 80% FEV1 in 1 second). UMEC inhibits acetylcholine binding and therefore opens the airways by inhibiting bronchoconstriction by blocking the M3 muscarinic receptor, which is abundantly expressed in the airway smooth lung muscle.

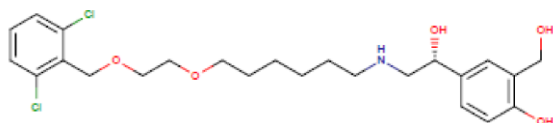


Fig 1(A): Chemical Structure of VI

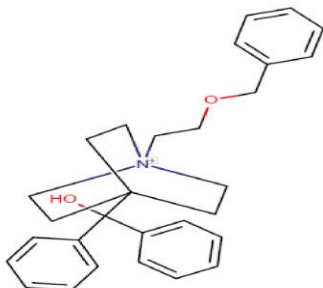


Fig 1(B): Chemical structure of UMEC

The literature survey conducted revealed some analytical studies[3-7] performed for the drugs, the key idea is to develop an easy, sensitive and accurate method for estimation of UMEC/VI in pharmaceutical dosage form employing RP-HPLC. In terms of linearity and range, precision, and accuracy, the suggested approach was verified using criteria from the International Conference on Harmonization [8-10].

2. EXPERIMENTAL

2.1 MATERIALS AND METHODS

2.1.1 Chromatographic Instrumentation: The analysis was performed using WATERS HPLC 2695 SYSTEM fitted with quaternary pumps, Photo Diode Array Detector and Auto Sampler integrated with Empower 2 software. Column used for separation was Phenomenex C₁₈ (150 x 4.6 mm, 5 μ). Ammonium acetate: acetonitrile taken in the ratio 60:40 as mobile phase and pumped through column at a flow rate of 0.9 ml/min at 30°C. Optimized wavelength selected was 245 nm.

2.1.2 Chemicals and reagent: HPLC grade Acetonitrile, Methanol, Distilled Water, Ortho – phosphoric acid buffer, Ammonium Acetate were obtained from Rankem. UMEC/VI pure drugs (API), UMEC/VI dosage form (Anoro Ellipta) were received from Spectrum Pharma Research Solution, Hyderabad.

2.1.3 Preparation of Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in 50:50 ratio

2.1.4 Preparation of Standard stock solutions: VI (2.5mg) and UMEC (6.25mg) were accurately weighed and added to a 10ml volumetric flask, added with 3/4th of the diluents, then sonicated for 10 minutes. Standard stock solution was prepared by filling flasks with diluents and labeled them as such. (VI (250 μ g/ml), UMEC (625 μ g/ml))

2.1.5 Preparation of Standard working solutions (100% solution): 1 ml of each stock solution was transferred into a 10 ml volumetric flask, which was then diluted. (VI (25 μ g/ml), UMEC (62.5 μ g/ml))

2.1.6 Preparation of sample stock solutions: In a 50 ml volumetric flask, the contents of nasal spray administered by 50 actuations (25 and 62.5 μ g each) were collected. Then 20ml acetonitrile was added, and it was sonicated for 25 minutes before being filled to the mark, yielding 1250 and 3125 μ g/ml. It was centrifuged for 20 minutes. Finally, the supernatant was collected and filtered through 0.45 μ m filters (Millipore, Milford and PVDF).

2.1.7 Preparation of sample working solution: 2ml of the sample stock solution was pumped into a 10ml volumetric flask, which was then filled with diluent. (VI (25 μ g/ml), UMEC (62.5 μ g/ml)).

2.2 Validation:

2.2.1 System suitability parameters:

The parameters, peak tailing, resolution and USP plate count were defined by preparing standard solutions of VI (25ppm) and UMEC (62.5ppm) and the solutions were injected six times.

2.2.2 Specificity:

In the presence of elements, specificity refers to the ability to measure the analyte unambiguously. In this approach we do not consider interfering peaks of these drugs in blank and placebo at retention times. So, the method is said to be accurate.

2.2.3 Precision:

The method was checked for both intra-day and inter-day precision.

2.2.4 Linearity:

Appropriate aliquots of UMEC/VI standard stock solutions were placed in separate 10 ml volumetric flasks and diluted up to the mark with mobile phases to achieve final concentrations of 15.625- 93.75µg/ml for UMEC and 6.25- 37.5µg/ml for VI. The solutions were then injected into the system and chromatograms were registered.

2.2.5 Accuracy:

Recovery trials were used to determine the method's accuracy. For this experiment, a standard addition approach was used. A known amount of UMEC and VI were introduced, equivalent to 50%, 100%, 150% of the drug's label claim, respectively. Each addition sets were performed three times. The accuracy was calculated as percentage of recovered analytes.

2.2.6 Robustness:

Tiny subtle improvements were made in methods such as flow rate, mobile phase ratio and temperature, but the effects have not been recognized and were within the limits of ICH Guidelines. Robustness conditions such as Flow minus (0.8ml/min), Flow plus (1.0ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) were established and samples were double-injected. System suitability parameters were not much affected and all parameters were passed. Percentage RSD was within the limit.

2.2.7 LOD:

The lowest amount of analyte that can be detected is referred to as the LOD. LOD values are derived from calibration curve using standard deviation (SD) and slope of calibration curve. The formula for determining LOD is,

$$\text{LOD} = 3.3 \times \text{avg SD/slope}$$

2.2.8 LOQ:

The smallest quantity of analyte that can be measured is referred as LOQ. LOQ is measured from calibration curve using standard deviation (SD) and slope of calibration curve. The formula for determining LOQ is,

$$\text{LOQ} = 10 \times \text{avg SD/slope}$$

2.3 Degradation studies:

Degradation studies were carried out by treating samples with acidic, alkaline, oxidative, hydrolytic, thermal condition and photolytic application. Acidic and alkaline degradation were carried using 2N HCl and 2N NaOH respectively. Oxidative degradation was performed using 20% H₂O₂ solution. Acidic, alkaline and oxidative degradation studies were kept at 60° C for 30 min. the stress testing within hydrolytic degradation were studied by refluxing the sample in H₂O for 1hr at 60°C. To study dry heat degradation, the standard drug solution was placed in the oven at 105 °C for 1 hr. The drug's photolytic degradation stability was also studied by exposing the 250µg/ml VI and 625µg/ml UMEC solution to UV Light by placing the beaker in the UV chamber for 1day or 200wt hours/m² in the photo stability chamber. The resulting solution were diluted 10 µl were injected into the system and the sample stability was evaluated by the chromatograms.

3. RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic Condition (Method Development)

Method development was done by changing various, mobile phase ratios, and buffers etc. methanol: water, methanol: OPA, acetonitrile: ammonium acetate in various ratios were tried as mobile phase. Finally, acetonitrile: ammonium acetate (40:60 v/v) was selected for the optimization of chromatographic condition. With reasonable resolution, UMEC/VI were elucidated at 2.219 min and 2.794 min. Optimized chromatogram is depicted in Fig 2.

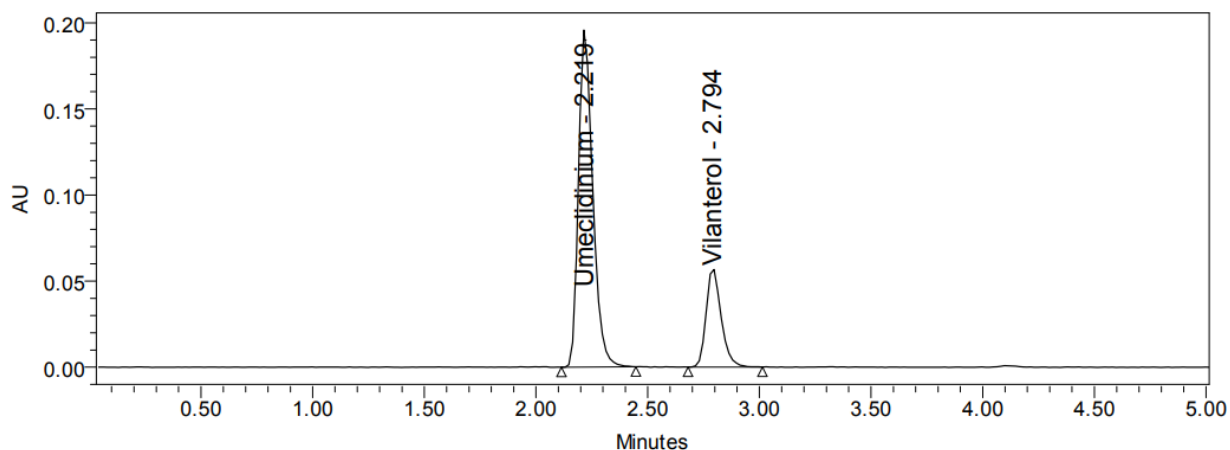


Fig 2: Optimized trial chromatogram of UMEC/VI

3.2. Method Validation

3.2.1 System suitability parameter

All system suitability parameters, peak tailing, resolution and USP plate count were within range according to ICH rules. The resolution was found to be 4.66. System suitability parameter and acceptance limit are shown in Table 1.

Table 1: system suitability parameter of UMEC/VI

Parameters	UMEC	VI	Acceptance limit
USP plate count	5595.5	8254	>2000
Tailing factor	1.256	1.206	<2
R _t (min)	2.219	2.794	≥2

3.2.2 Specificity

No intrusive peaks at retention time of main drug were observed after injecting blank and placebo, so the method was said to specific. Chromatograms are depicted in Fig. 3-5.

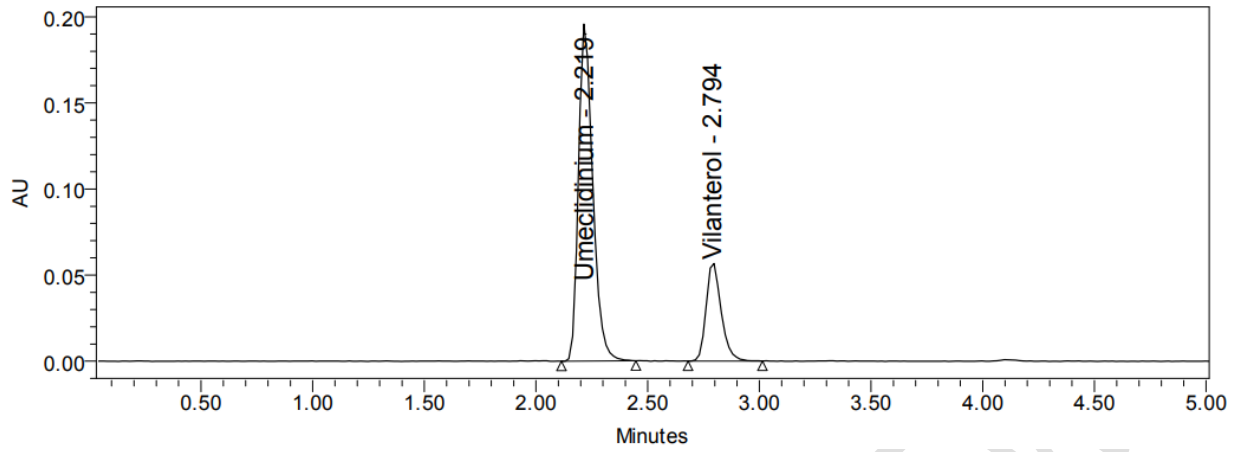


Fig 3: Optimized Chromatogram of UMEC/VI

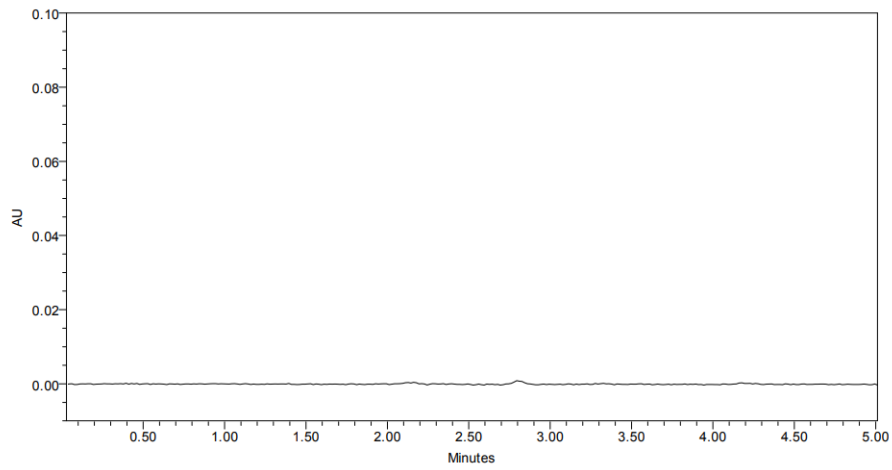


Fig 4: Chromatogram of Blank

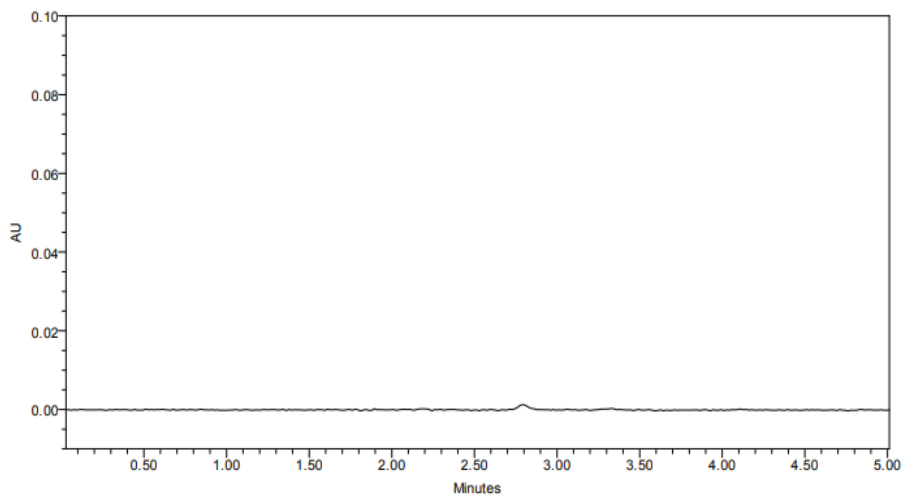


Fig 5: Chromatogram of Placebo

3.2.3 Precision

3.2.3.1 System precision

System precisions of the drug UMEC/VI were obtained using six injections from the flask of standard working solution. Percentage RSD was found to be 1.0 and 0.9 for UMEC/VI. Average mean, standard deviation, %RSD, area were depicted in Table 2. Since the %RSD value is below the limit, the system precision was passed.

Table 2: System Precision data of UMEC/VI

S. No	Area of UMEC	Area of VI
1.	1044088	310896
2.	1068906	312810
3.	1038946	304680
4.	1053004	309307
5.	1056861	309562
6.	1045400	310015
Mean	1051201	309545
S.D	10799.9	2699.3
%RSD	1.0	0.9

3.2.3.2 Repeatability (Intraday Precision)

Multiple samples were taken from a sample stock solution, and six working sample solutions of the same concentration were produced. Each injection from each working sample solution was administered, and the results were collected and expressed in Table 3. Percentage RSD was found to be 0.8 and 0.6 for UMEC/VI.

Table 3: Intraday Precision data of UMEC/VI

S. No	Area of UMEC	Area of VI
1.	1052473	311121
2.	1062990	313699
3.	1052423	310257
4.	1037911	315766
5.	1045980	312796
6.	1045358	312044

Mean	1049523	312614
S.D	8523.0	1962.4
%RSD	0.8	0.6

3.2.3.3 Interday Precision

Multiple samples were taken from a sample stock solution and six working sample solutions of the same concentration were prepared. Each injection from each working sample solution was administered on next day of preparation, and the results were collected and expressed in Table 4. Percentage RSD was found to be 1.6 and 1.1 for UMEC/VI.

Table 4: Interday precision data of UMEC/VI

S. No	Area of UMEC	Area of VI
1.	937737	296281
2.	934051	301399
3.	924577	304606
4.	959381	305116
5.	934412	301443
6.	962788	300862
Mean	942158	301618
S.D	15340.1	3171.7
%RSD	1.6	1.1

3.2.4 Linearity

The method was linear over the range 15.625 - 93.75µg/ml and 6.25 - 37.5µg/ml for UMEC/VI. The calibration curve was constructed by plotting the response factor against a concentration of drugs (Fig 6, Fig 7). The slope and intercept value for calibration curve was $y = 16315x + 10672$, $R^2 = 0.9992$ for UMEC and $y = 12261x + 4146.2$, $R^2 = 0.999$ for VI. The results show an excellent correlation between the response factor and the concentration of drugs. Data depicted in Table 5.

Table 5: Linearity data of UMEC/VI

UMEC		VI	
Conc (µg/ml)	Peak area	Conc (µg/ml)	Peak area
0	0	0	0
15.625	260485	6.25	79061
31.25	520706	12.5	157844
46.875	791492	18.75	239800
62.5	1055629	25	319198
78.125	1275611	31.25	384674
93.75	1524272	37.5	457694

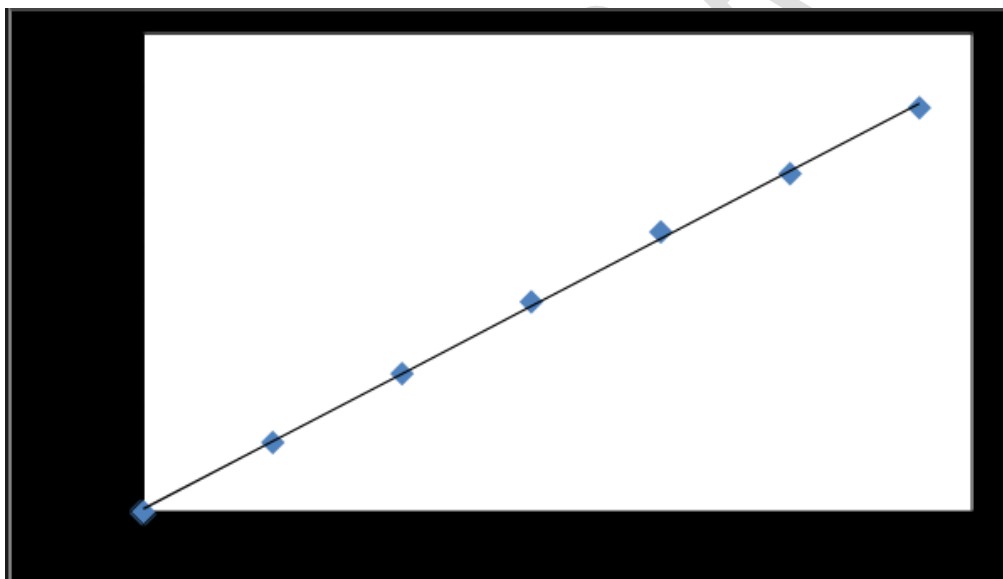


Fig 6: Calibration curve of UMEC

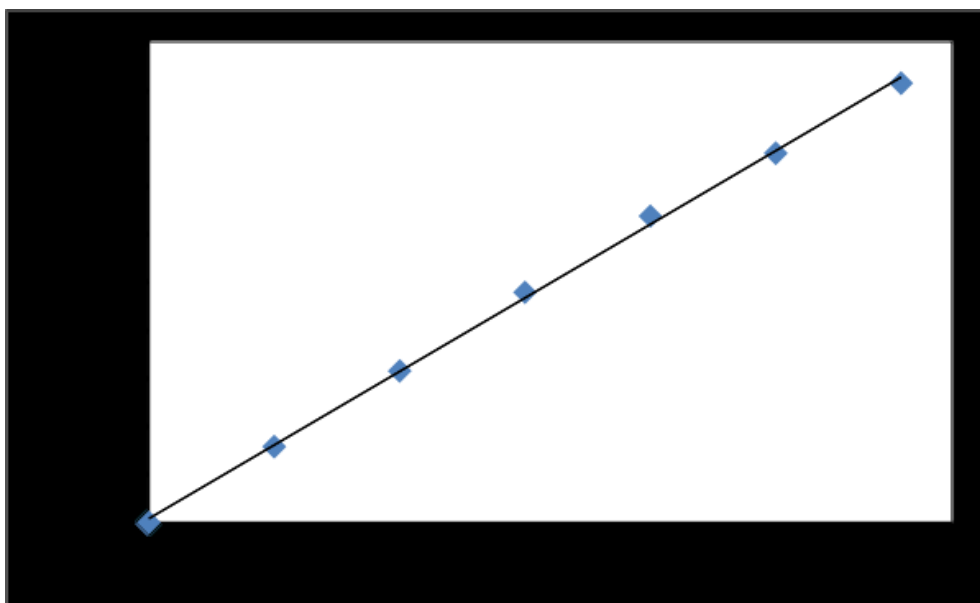


Fig 7: Calibration curve of VI

3.2.5 Accuracy

The approach's accuracy was tested three times using the conventional addition technique, each time in triplicate. The proportion of standard drug recovered by the recovery study was then used to calculate the accuracy. **Table 6** shows the mean UMEC/VI recovery rates from the combined formulation. Accuracy is measured in terms of % Recovery. Percentage Recovery was obtained as 100.09% and 99.94% for UMEC/VI.

Table 6: Accuracy data of proposed study of UMEC/VI

% level	UMEC			VI		
	Amount Spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	Amount Spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery
50%	31.125	31.13	100.03	1.25	1.24	99.11
	31.125	31.33	100.67	1.5	1.25	100.34
	31.125	31.38	100.83	1.25	1.27	101.62
100%	62.5	62.78	100.45	2.5	2.49	99.75
	62.5	62.65	100.23	2.5	2.45	98.19
	62.5	62.46	99.93	2.5	2.47	99.00

	93.75	93.57	99.81	3.75	3.78	100.73
150%	93.75	92.87	99.06	3.75	3.79	101.17
	93.75	93.53	99.77	3.75	3.73	99.59
Mean						
%		100.09%			99.94%	
Recovery						

3.2.6 Robustness

Robustness of the method was determined by changing three HPLC variables, like flow rate (0.8 and 1.0 ml min⁻¹), mobile phase ratio (65B: 35A and 55B:45A) and column temperature (25 and 35°C). The effects of these changes on the % RSD of both the substances (UMEC/VI) were determined. The value of percentage standard deviation was within the range, as shown in Table 7, which indicates that the developed method is robust and none of the variables possess significant effects on the method performance.

Table 7: Robustness Data of UMEC/VI

Parameter	Condition	% RSD	
		UMEC	VI
Change in flow rate (± 0.1 ml/ min)	0.8 ml min ⁻¹	1.1	1.4
	1.0 ml min ⁻¹	0.7	0.7
Change in mobile phase composition acetonitrile: ammonium acetate (± 5 ml)	65B:35A	0.6	0.4
	55B:45A	0.4	0.3
Change in column temperature (±5 °C)	25 °C	1.1	1.6
	35°C	1.2	1.0

3.2.7 LOD and LOQ

Table 8 shows the minimum detection limit and minimum quantitation limit of UMEC/VI. LOD of UMEC/VI were found to be 0.33 and 0.15. LOQ of UMEC/VI were found to be 1.01 and 0.45, respectively.

Table 8: LOD and LOQ Data of UMEC/VI

Molecule	LOD	LOQ
UMEC	0.33	1.01
VI	0.15	0.45

3.2.8 Assay:

The label containing **Anoro Ellipta** specifies UMEC 62.5 mcg, VI 25mcg. Assay with dosage type was performed. The mean percentage of assays obtained for UMEC and VI were 99.64 and 100.79 percent respectively. Assay data is depicted in Table 9 and Table 10.

Table 9: Assay data of UMEC

S.no	Standard Area	Sample area	% Assay
1	1044088	1052473	99.92
2	1068906	1062990	100.92
3	1038946	1052423	99.92
4	1053004	1037911	98.54
5	1056861	1045980	99.30
6	1045400	1045358	99.25
Avg	1051201	1049523	99.64
SD	10799.9	8523.0	0.81
%RSD	1.0	0.8	0.8

Table 10: Assay data of VI

S.no	Standard Area	Sample area	% Assay
1	310896	311121	100.31
2	312810	313699	101.14
3	304680	310257	100.03
4	309307	315766	101.81
5	309562	312796	100.85
6	310015	312044	100.61
Avg	309545	312614	100.79
SD	2699.3	1962.4	0.6
%RSD	0.9	0.6	0.6

3.3 Degradation studies

Acidic, alkaline, oxidative, thermal, photolytic and hydrolytic degradation studies were performed on UMEC/VI. Since the percentage degradation was under acceptance criteria, it represents the stability-indicating properties of the method. Percentage degradation was demonstrated in Table 11. The chromatograms of the sample are shown in Fig 8 – 13.

Table 11: Degradation study data of UMEC/VI

Stress condition	UMEC			VI		
	Area	%Recovered	% Degraded	Area	%Recovered	% Degraded
Acidic	992163	94.20	5.80	292294	94.24	5.76
Alkaline	999681	94.91	5.09	294629	94.99	5.01
Oxidative	1006898	95.59	4.41	296282	95.52	4.48
Thermal	1030374	97.82	2.18	302182	97.43	2.57
Photolytic	1039498	98.69	1.31	304757	98.26	1.74

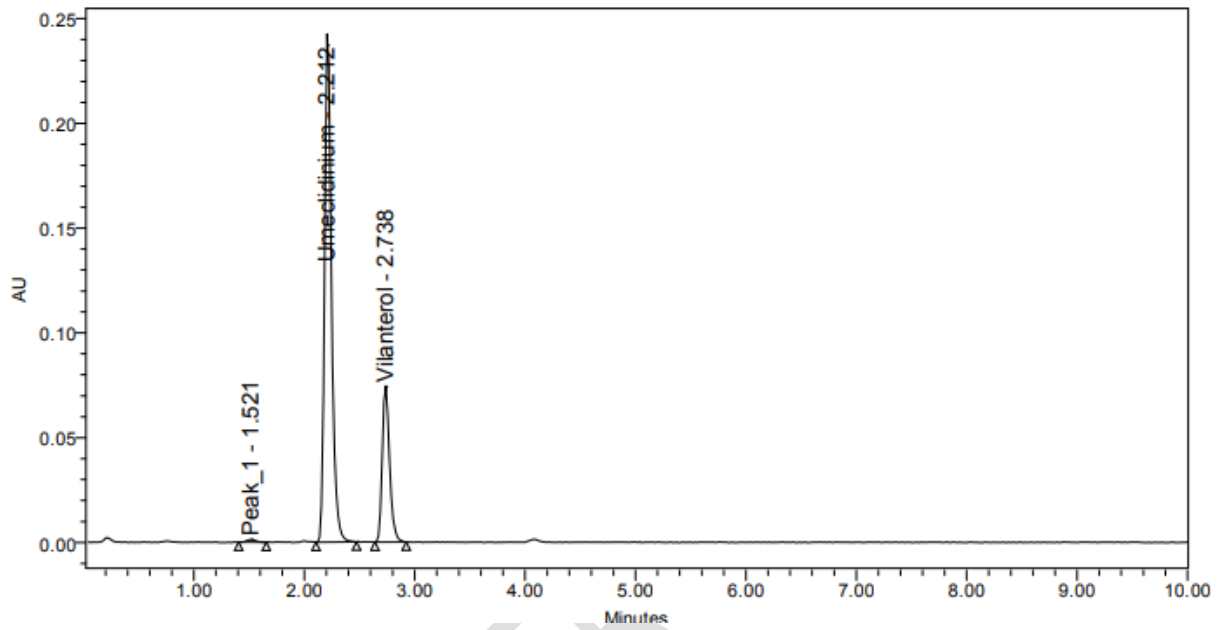


Fig 8: Acidic degradation chromatogram of UMEC/VI

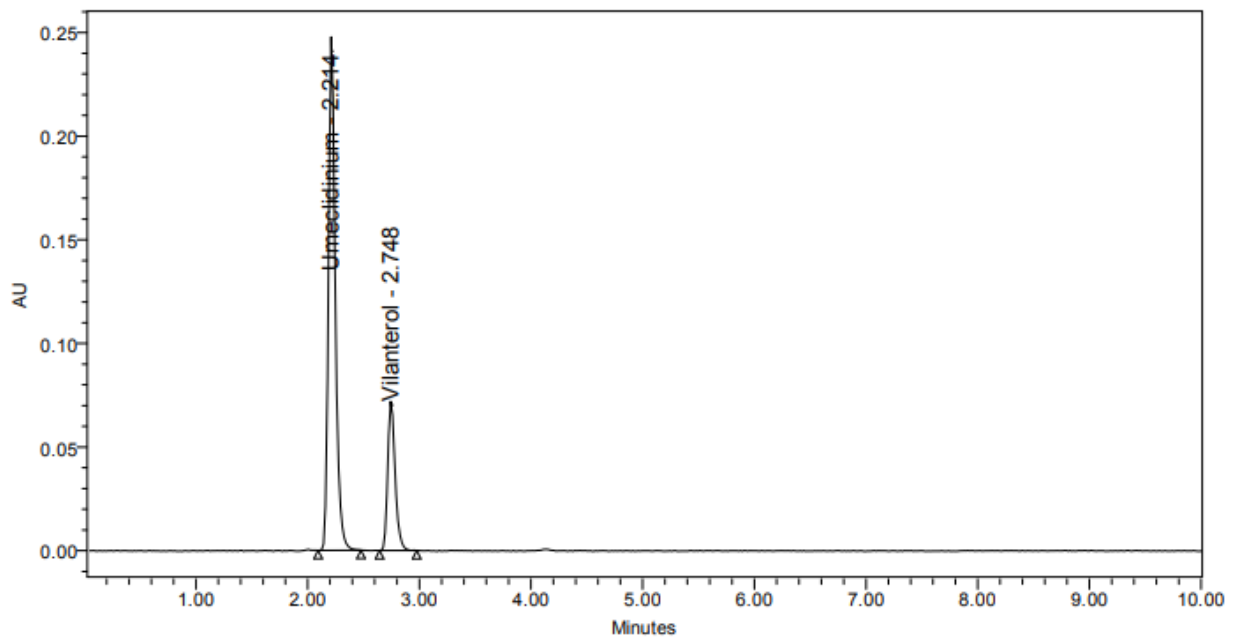


Fig 9: Alkaline degradation chromatogram of UMEC/VI

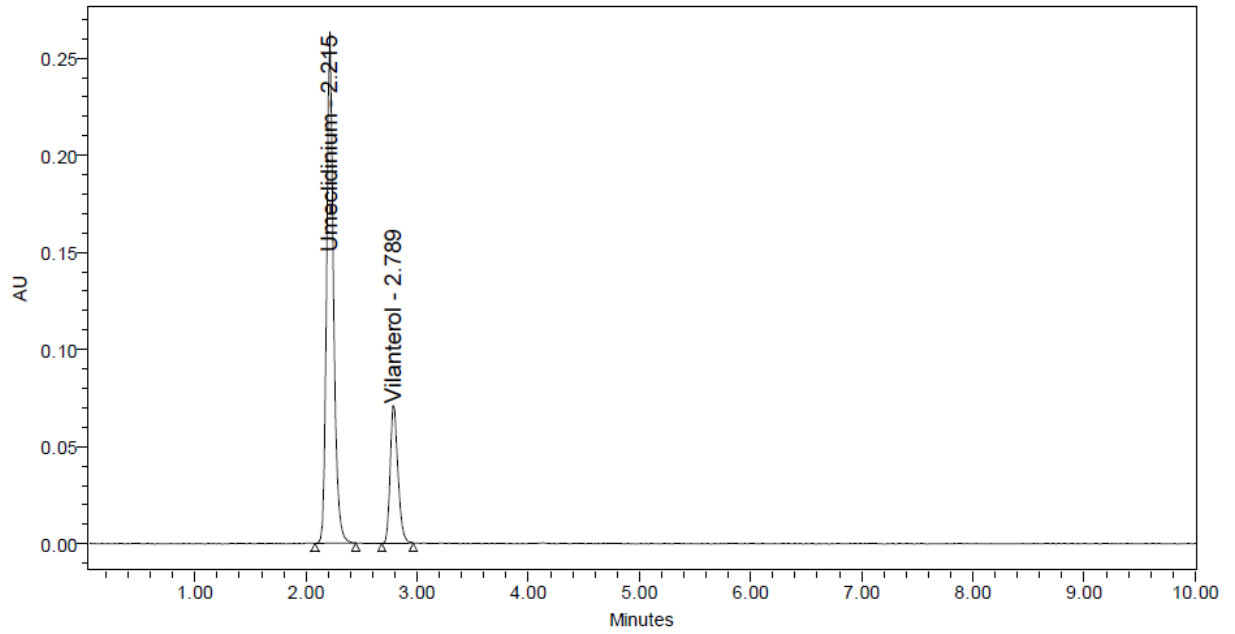


Fig 10: Oxidative degradation chromatogram of UMEC/VI

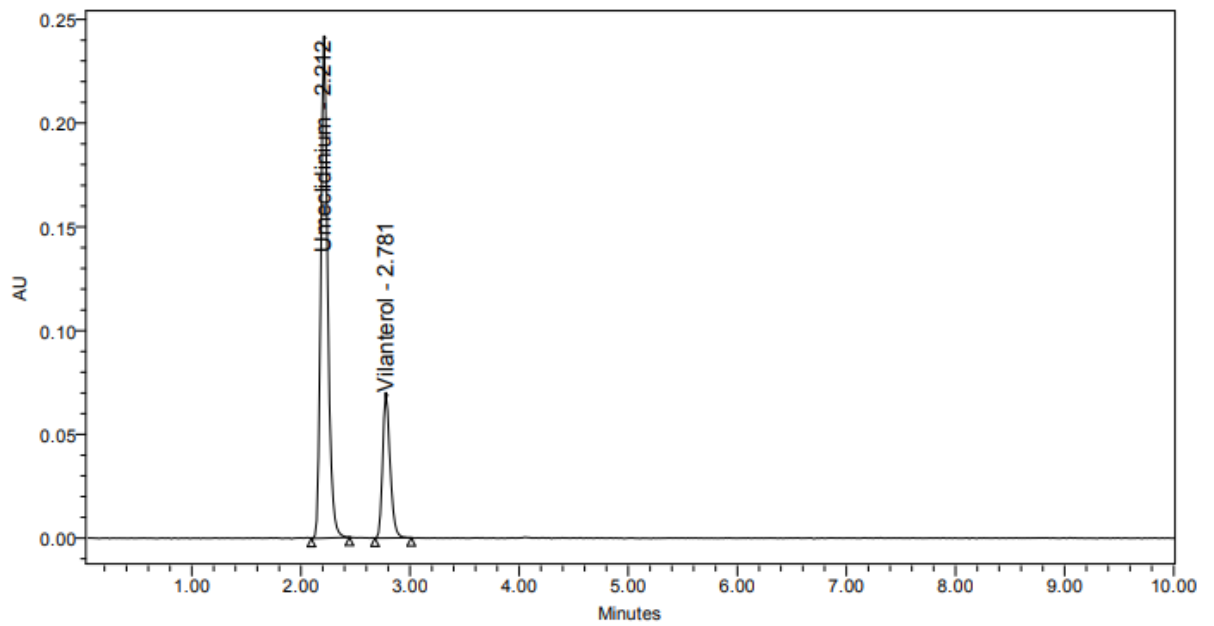


Fig 11: Thermal degradation chromatogram of UMEC/VI

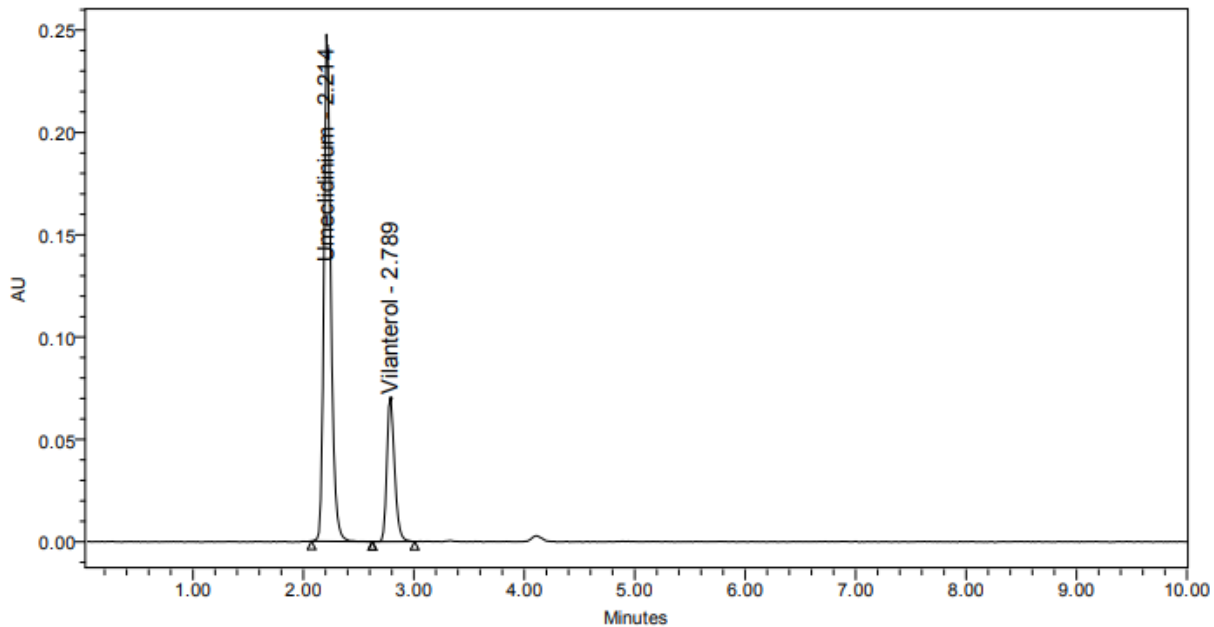


Fig 12: Photolytic degradation chromatogram of UMEC/VI

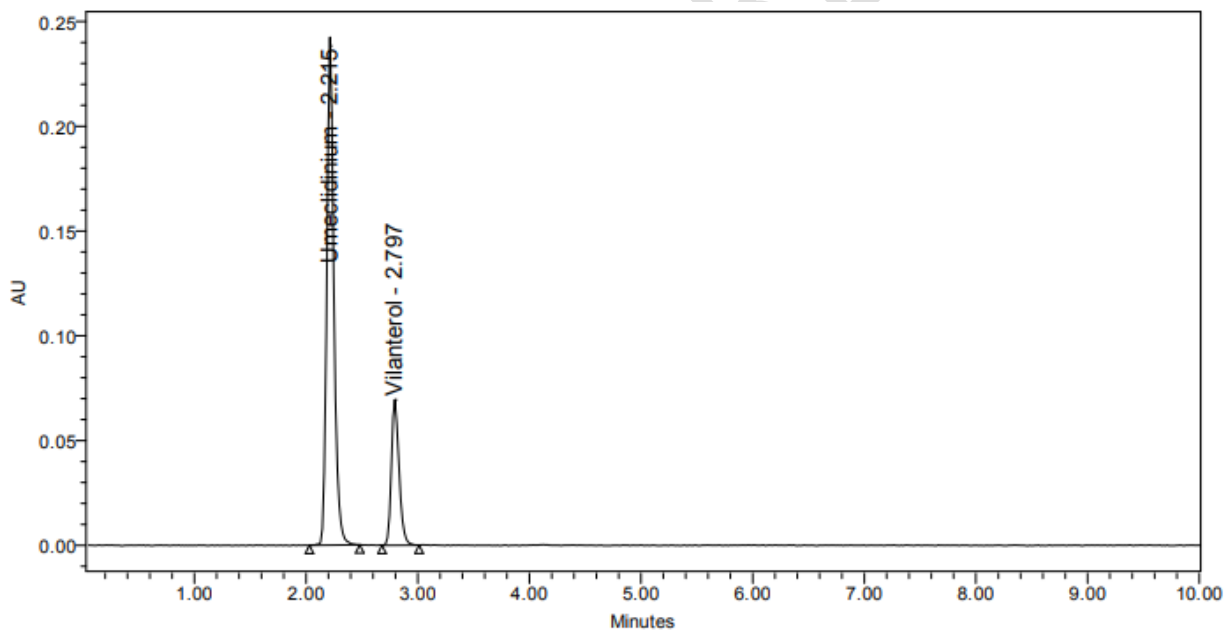


Fig 13: Hydrolytic degradation chromatogram of UMEC/VI

4. CONCLUSION

For the simultaneous determination of UMEC/VI in powder dosage form a simple, linear, sensitive and accurate method was developed. The method utilized Phenomenex C_{18} column, Acetonitrile: ammonium acetate taken in 40:60(v/v) ratio was taken as mobile phase was pumped at a flow rate 0.9ml/min at 30°C. Detection wavelength selected was

245 nm. Retention times of Umeclidinium and Vilanterol were found to be 2.219 min and 2.794 min. % RSD of UMEC/VI were found to be 1.0 and 0.9. Percentage Recovery was obtained as 100.09% and 99.94% for UMEC/VI respectively. LOD, LOQ values obtained for UMEC/VI were 0.33, 1.01 and 0.15, 0.45 respectively. Regression equation for UMEC was $y = 16315x + 10672$, and $y = 12261x + 4146.2$ of VI. The mean percentages of assay for UMEC/VI were obtained as 99.64% and 100.79%. Validation of the method developed was performed as per the ICH Q2 (R1) guideline. The percentage of degradation resulted from the stability studies were found to be under the acceptance limit. The method developed was low cost, easy, and sensitive and fast that can be adopt in regular Quality control test in companies.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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