RESEARCH PAPER

Development and Validation of a Stability-Indicating RP-UPLC Method for Estimation of Brexanolone in Pharmaceutical Dosage Forms

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Abstarct:
An Easy, sensitive, specific, and precise RP-UPLC method for the pharmaceutical dose estimation of Brexanolone in tablet dosage form. Chromatogram was run through ACQUITY UPLC HSS C18 Column, 50, 1.8 µm, 2.1. Mobile phase containing 0.1% OPA : MeCN taken in the ratio 70:30 was pumped through column at a flow rate of 0.3ml/min. Temperature was maintained at 30°C. Optimized wavelength selected was 235.0nm. Retention time of Brexanolone was found to be 0.907 min. %RSD of the Brexanolone were and found to be 0.5. %RSD of Method precision of Brexanolone was found to be 0.3%. %Recovery was obtained as 99.59% for Brexanolone. LOD, LOQ values obtained from regression equation of Brexanolone were 0.08, 0.25. Regression equation of Brexanolone is y = 64349x + 1674.7. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.
Keywords: Brexanolone, RP-UPLC, Method Development, Method Validation, Pharmaceutical Analysis, Stability Indicating Method, ICH Guidelines, Chromatography.
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INTRODUCTION

Pharmaceutical analysis plays a vital role in ensuring the quality, safety, and efficacy of drug substances and pharmaceutical dosage forms. Accurate analytical methods are essential for the identification, quantification, purity assessment, and stability evaluation of active pharmaceutical ingredients (APIs). Among the various analytical techniques available, chromatographic methods are widely accepted because of their sensitivity, specificity, reproducibility, and ability to separate complex mixtures effectively (Snyder et al., 2012).

Chromatography has become one of the most important tools in pharmaceutical and bioanalytical research. It enables the separation and estimation of compounds even in the presence of impurities, degradation products, excipients, and biological matrices. High performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are extensively employed for routine quality control, stability studies, dissolution testing, pharmacokinetic studies, and method validation (Dong, 2019).

UPLC is an advanced form of liquid chromatography developed to overcome the limitations of conventional HPLC systems. The technique utilizes columns packed with very small particle sizes and operates at higher pressures, resulting in enhanced chromatographic performance. UPLC offers several advantages such as improved resolution, reduced analysis time, increased sensitivity, lower solvent consumption, and better peak capacity (Swartz, 2005). These characteristics make UPLC highly suitable for rapid and efficient pharmaceutical analysis.

The growing demand for faster analytical methods with higher efficiency has increased the application of UPLC in pharmaceutical industries

and research laboratories. UPLC methods are particularly useful for the simultaneous estimation of multiple drugs, impurity profiling, degradation studies, and bioanalytical estimations. The technique supports high-throughput analysis while maintaining excellent reproducibility and robustness (Kazakevich & Lobrutto, 2007).

Analytical method development and validation are important steps in chromatographic analysis to ensure the reliability and consistency of the obtained results. Validation parameters such as specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantification are evaluated according to International Council for Harmonisation (ICH) guidelines (ICH Q2(R1), 2005). A properly validated chromatographic method can be effectively utilized for routine quality control and regulatory submissions.

Brexanolone is a neuroactive steroid and synthetic formulation of allopregnanolone used for the treatment of postpartum depression (PPD). It acts as a positive allosteric modulator of GABA-A receptors, enhancing inhibitory neurotransmission in the central nervous system. Brexanolone possesses the molecular formula C21H34O2 and molecular weight of 318.49 g/mol. The drug is administered through continuous intravenous infusion and exhibits rapid antidepressant activity compared with conventional therapies. It undergoes extensive metabolism by glucuronidation, sulfation, and keto-reduction pathways with minimal involvement of cytochrome P450 enzymes. Common adverse effects include dizziness, sedation, and dry mouth. Due to its rapid therapeutic response and unique mechanism of action, brexanolone has gained significant importance in pharmaceutical and bioanalytical research, particularly in chromatographic method development and UPLC-based estimation studies.

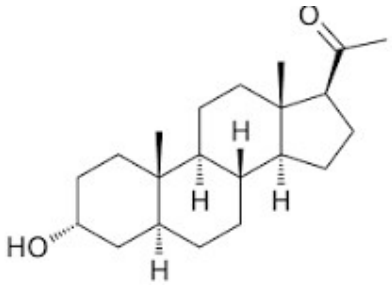

Figure 1. Chemical Strucure of Brexanolone

Materials and Instruments Used

The materials used for the present study included analytical and UPLC grade reagents and solvents to ensure accuracy and reproducibility of the chromatographic analysis. Acetonitrile, water, and methanol of UPLC grade were utilized as mobile phase components and diluents. Potassium dihydrogen orthophosphate and orthophosphoric acid of analytical reagent (AR) grade were employed for the preparation and adjustment of buffer solutions. A 0.2 µm Millipore membrane filter was used for filtration of mobile phase and sample solutions prior to UPLC analysis to remove particulate matter and maintain system suitability.

The analytical study was carried out using advanced laboratory instruments and equipment. UV spectrophotometric analysis was performed using a Microprocessor UV-Visible Single Beam Spectrophotometer. Chromatographic separation and estimation were achieved using an Acquity UPLC system equipped with a Tunable UV (TUV) detector. Sample weighing was carried out using a Sartorius Scaletec BSA224S-CW analytical balance to ensure precise measurement of chemicals and standards. Ultrasonication was performed using a Lab Man ultrasonicator for proper dissolution and degassing of solutions. The pH of buffer solutions was adjusted using a Lab Man pH meter. A Sisco hot air oven was utilized for drying purposes, while a Remi vortex mixer was employed for uniform mixing of sample solutions during preparation.

The marketed pharmaceutical formulation used in the present investigation was Zulresso injection containing brexanolone with a labeled claim of 5 mg/mL. The formulation was procured from the local market and used for method development and analytical validation studies.

METHODS

Diluent: Based up on the solubility of the drugs, diluent was selected, Methanol and Water taken in the ratio of 60:40

Preparation of Buffer:

0.1% Trifluoracetic acid buffer: 1ml of TFA solution was diluted to 1000ml with UPLC grade water.

Preparation of Standard stock solutions: Accurately weighed 5mg of Brexanolone transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (100µg/ml solution of Brexanolone)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (10µg/ml of Brexanolone).

Preparation of Sample stock solutions: 1vial of injection containing 5mg/ml sample was taken into a 50ml volumetric flask, 3/4thml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by UPLC filters (100µg/ml of Brexanolone)

Preparation of Sample working solutions (100% solution): 1.0ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (10µg/ml of Brexanolone).

Validation:

System suitability parameters:

The system suitability parameters were determined by preparing standard solution of Brexanolone (10ppm) and the solution were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Standard stock solutions: Accurately weighed 5mg of Brexanolone transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (100µg/ml solution of Brexanolone)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (10µg/ml of Brexanolone).

The test solution was determined by preparing test solution of Brexanolone (40ppm) and the solution were injected six times and the % RSD for the area of six standard injections results should not be more than 2%.

Linearity:

Preparation of Standard stock solutions: Accurately weighed 5mg of Brexanolone transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (100µg/ml solution of Brexanolone)

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (2.5µg/ml of Brexanolone)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (5µg/ml of Brexanolone)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (7.5µg/ml of Brexanolone)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (10µg/ml of Brexanolone)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Brexanolone)

150% Standard solution: 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml (15µg/ml of Brexanolone)

Accuracy:

Preparation of Sample stock solutions: 1vial of injection containing 5mg/ml sample was taken into a 50ml volumetric flask, 3/4thml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by UPLC filters (100µg/ml of Brexanolone)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml

volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.27ml/min), Flow plus (0.33ml/min), mobile phase minus, mobile phase plus, temperature minus (27°C) and temperature plus (33°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Brexanolone, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each for Brexanolone, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:

Oxidation:

To 1 ml of stock solution of Brexanolone, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 600c. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stock solution Brexanolone, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 600c. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Brexanolone, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 600c. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105°C for 6h to study dry heat degradation. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 100µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200-Watt hours/m2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For UPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 1µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

RESULTS AND DISCUSSION

Initial UPLC runs of Brexanolone

Initial UPLC runs of Brexanolone of 10µg/ml concentration were performed using

- Different buffer viz, Potassium dihyrogen ortho phosphate and Ortho phosphoric acid.
- Different organic modifier viz, acetonitrile and methanol
- Different columns such as ACQUITY UPLC HSS PFP Columns, ACQUITY CHS Column, 300Å, 1.7 µm, 2.1 mm x 150 mm, ACQUITY BEH Column, 300Å, 1.7 µm, 2.1 mm x 50 mm) ACQUITY UPLC CHS Column, 300Å, 1.7 µm, 2.1 mm X 150 mm, ACQUITY UPLC and ACQUITY UPLC Peptide HSS T3 Column, 100Å, 1.8 µm, 1 mm X 100 mm

Authentication and identification of received API

Reported Solubility Characteristics

- **Water:** Practically insoluble / very slightly soluble (approx 0.00136 mg/mL)
- **Methanol:** Freely soluble
- **Ethanol:** Soluble
- **Acetonitrile:** Soluble

- **Dimethyl sulfoxide (DMSO):** Freely soluble

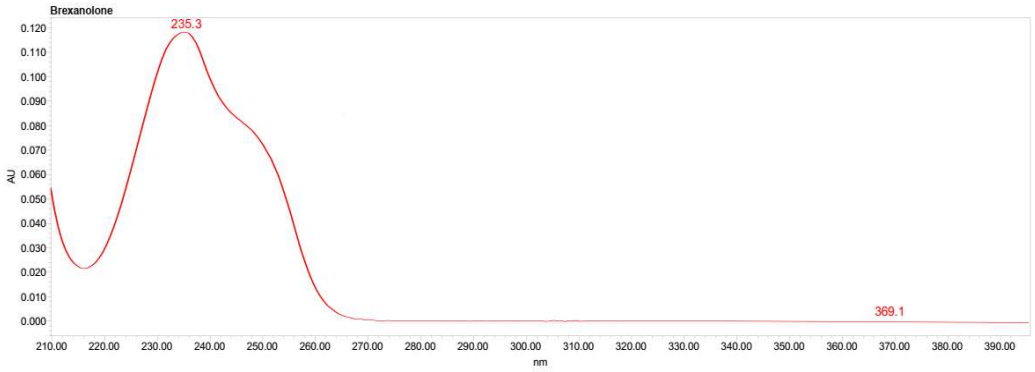

Figure 2: UV Spectrum of Brexanolone in methanol Solvent

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Total Three factors viz; % Organic concentration, Flow rate and column temperature were needed to be optimized. So, CCD was used to optimize these parameters which were varied over three level (high, mid, and low). different ranges of four parameters ranging from 25-35% of Buffer (Organic phase), column temperature 27 and 33^{0}C and 0.27-0.33ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, Area, Theoretical plates, and Asymmetry (CQA) were generated. A desirability function applied to the optimized conditions to predict retention time, asymmetry, theoretical plates,

Optimized condition

Mobile phase: MeCN: 0.1% OPA(30:70)
Flow rate: 1.0 ml/min
Column: HSS 250mm x 4.6 mm, 5µ.
Detector wave length: 235 nm
Column temperature: 30°C
Injection volume: 1µL
Run time: 2 min
Diluent: Water and Acetonitrile in the ratio 50:50
Results: peak have good resolution, tailing factor, theoretical plate count and resolution.

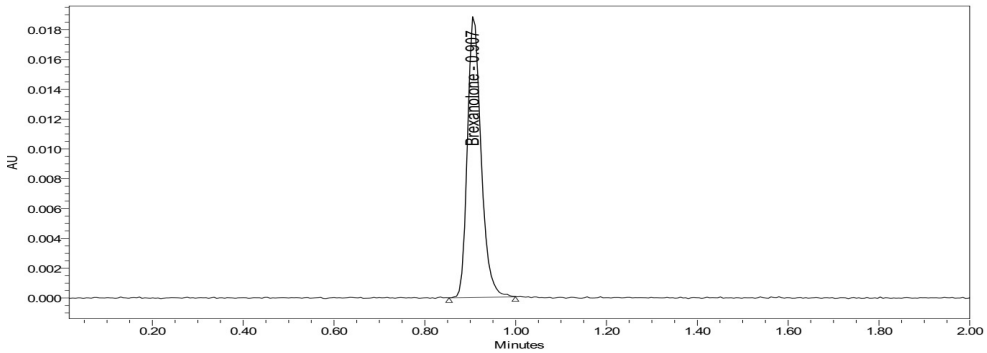

Figure 3: Optimized Chromatogram of Brexanolone

Observation: Finerenone were eluted at 0.907 min with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guideline

Table: 1 System suitability parameters for Brexanolone

S no	Brexanolone		
Inj	RT(min)	USP Plate Count	Tailing

1	0.855	2991	1.22
2	0.859	2921	1.21
3	0.859	2928	1.21
4	0.870	2990	1.22
5	0.871	3001	1.22
6	0.872	3019	1.22

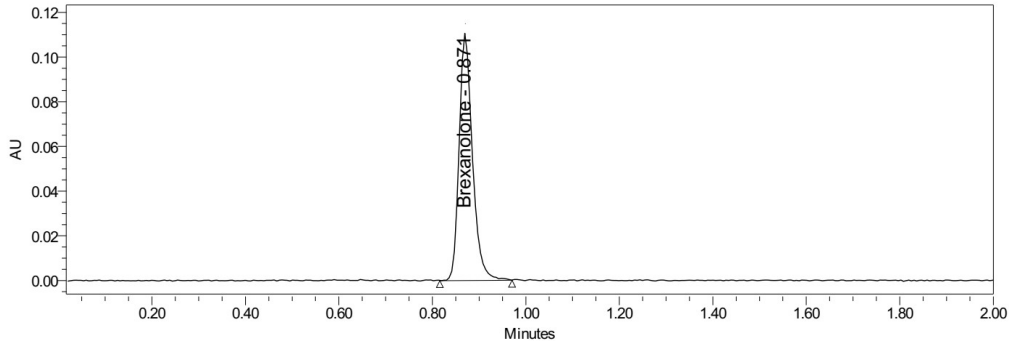

Figure 4 System suitability Chromatogram

Discussion: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Validation:

Specificity:

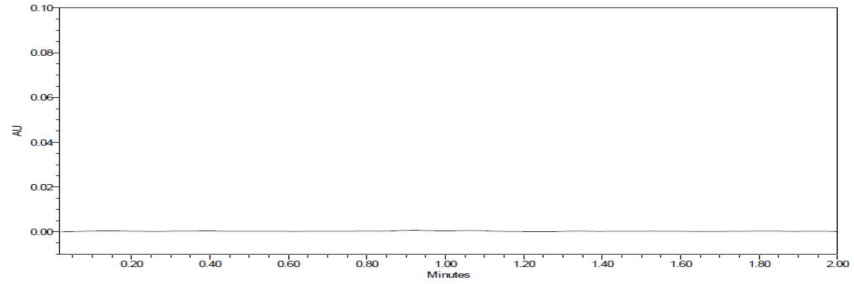

Figure 5 Chromatogram of blank

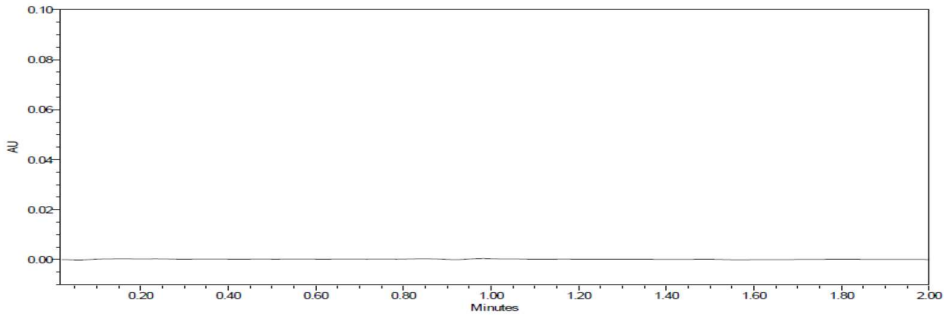

Figure 6. Chromatogram of placebo

Linearity:

Table 2 Linearity table for Brexanolone

Brexanolone	
Conc (µg/mL)	Peak area
0	0

2.5	160670
5	328891
7.5	482760
10	648400
12.5	801694
15	967621

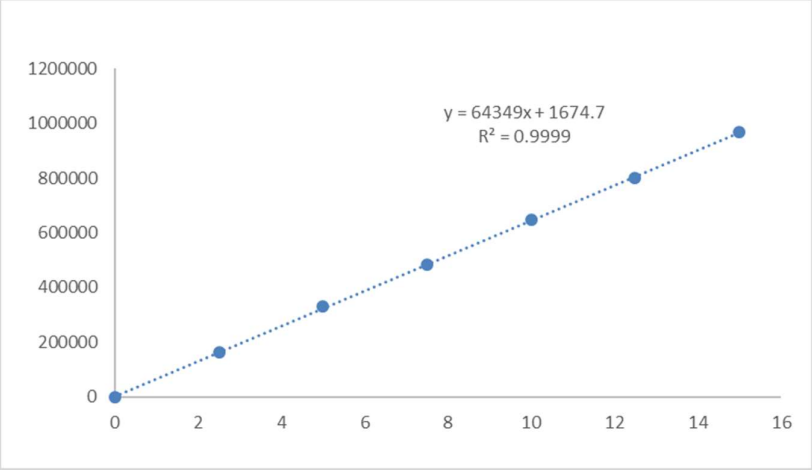

Figure 7 Calibration curve of Brexanolone

Discussion: Six linear concentrations of Brexanolone (2.5-15µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Brexanolone was y = 64349x + 1674.7. Correlation coefficient obtained was 0.999 for the two drugs.

Precision: System Precision:

Table 3. System precision table of Brexanolone

S. No	Area of Brexanolone
1.	641143
2.	637833
3.	638524
4.	640541
5.	639019
6.	646670
Mean	640622
S.D	3212.7
%RSD	0.5

Discussion: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated. % RSD obtained as 0.5% for Brexanolone. As the limit of Precision was less than "2" the system precision was passed in this method.

Repeatability:

Table 4. Repeatability table of Brexanolone

S. No	**Area of Brexanolone**
1.	636995
2.	634943
3.	634626

4.	639661
5.	637233
6.	639550
Mean	637168
S.D	2160.3
%RSD	0.3

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated and obtained as 0.3% for Brexanolone. As the limit of Precision was less than "2" the system precision was passed in this method.

Intermediate precision (Day _Day Precision):

Table 5. Intermediate precision table of Brexanolone

S. No	Area of Brexanolone
1.	500760
2.	495523
3.	500817
4.	495845
5.	500089
6.	504465
Mean	499583
S.D	3389.8
%RSD	0.7

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated and obtained as 0.7% for Brexanolone. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy:

Table 6. Accuracy table of Brexanolone

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	5	4.96	99.20	99.59%
	5	4.97	99.31	
	5	4.99	99.70	
100%	10	9.95	99.52	
	10	9.98	99.81	
	10	9.98	99.77	
150%	15	14.99	99.95	
	15	14.98	99.86	
	15	14.88	99.20	

Discussion: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.59% for Brexanolone.

Sensitivity:

Table 7. Sensitivity table of Brexanolone

Molecule	LOD	LOQ
Brexanolone	0.08	0.25

Robustness:

Table 8. Robustness data for Brexanolone

S.no	Condition	%RSD of Brexanolone
1	Flow rate (-) 0.27ml/min	1.0
2	Flow rate (+) 0.33ml/min	0.8
3	Mobile phase (-) 75B:25A	0.4
4	Mobile phase (+) 85B:15A	0.3
5	Temperature (-) 22°C	0.6
6	Temperature (+) 32°C	0.4

Discussion: Robustness conditions like Flow minus (0.27ml/min), Flow plus (0.33ml/min), mobile phase minus (75:25A), mobile phase plus (85:15A), temperature minus (27°C) and temperature plus (33°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

$$\%\text{Assay} = \frac{\text{Sample Peak Area}}{\text{Standard Peak Area}} \times 100$$

Assay: Average % Assay for Brexanolone obtained was 99.36%

Table 9. Assay Data of Brexanolone

S.no	Standard Area	Sample area	% Assay
1	641143	636995	99.3
2	637833	634943	99.0
3	638524	634626	99.0
4	640541	639661	99.8
5	639019	637233	99.4
6	646670	639550	99.7
Avg	640622	637168	99.36
Stdev	3212.7	2160.3	0.34
%RSD	0.5	0.3	0.3

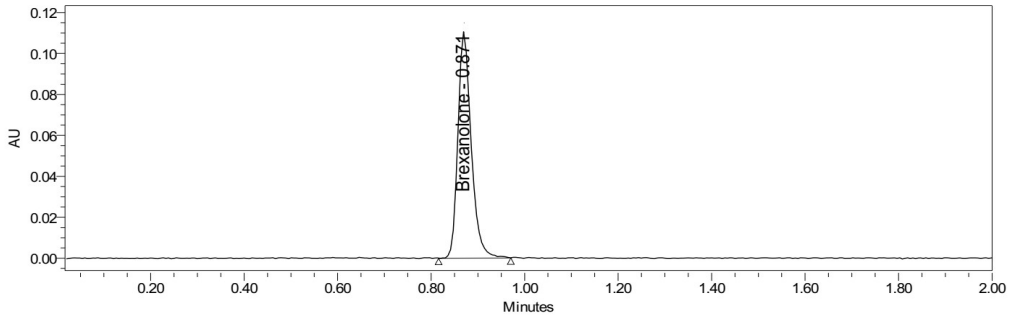

Figure 8. Chromatogram of working standard solution

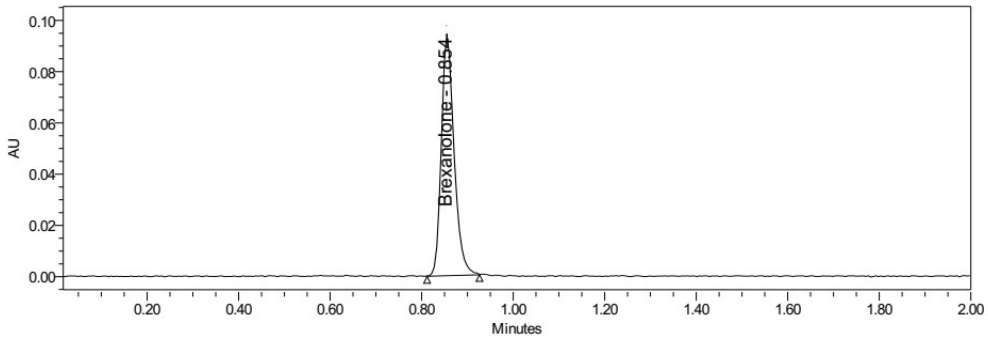

Figure 9. Chromatogram of working sample solution

DEGRADATION

Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation

Table 10. Degradation Data of Brexanolone

S.NO	Degradation Condition	% Drug un Degraded	% Drug Degraded
1	Acid	94.40	5.60
2	Alkali	94.74	5.26
3	Oxidation	92.46	7.54
4	Thermal	96.51	3.49
5	UV	97.69	2.31
6	Water	99.12	0.88

Discussion: Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time.

Summary

Table 11. Summary results of Brexanolone

Parameters		Brexanolone	Limit
Linearity Range(µg/ml)		2.5-15µg/ml	R< 1
Regression co-efficient		0.999	
Slope(m)		64349	
Intercept(c)		1674.7	
Regression equation (Y=mx+c)		y = 64349x + 1674.7	
Assay (% mean assay)		99.36%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		0.5	NMT 2.0%
Method precision %RSD		0.3	NMT 2.0%
Accuracy %recovery		99.59%	98-102%
LOD		0.08	NMT 3
LOQ		0.25	NMT 10
Robustness	**FM**	1.0	%RSD NMT 2.0
	FP	0.8	
	MM	0.4	
	MP	0.3	
	TM	0.6	

	TP	0.4	

CONCLUSION

A Simple, sensitive, specific, and precise UPLC method for the Determination of Brexanolone in API and pharmaceutical dosage form. Retention time of Brexanolone was found to be 0.907 min. %RSD of the Brexanolone were and found to be 0.5. %RSD of Method precision of Brexanolone was found to be 0.3%. %Recovery was obtained as 99.59% for Brexanolone. LOD, LOQ values obtained from regression equation of Brexanolone were 0.08, 0.25. Regression equation of Brexanolone is y = 64349x + 1674.7. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Justification for Selection of International Journal of Drug Delivery Technology (IJDDT)

International Journal of Drug Delivery Technology (IJDDT) was selected for publication because the journal focuses on pharmaceutical analysis, analytical method development, chromatographic validation, and quality control studies. The present work involving RP-UPLC method development and validation for estimation of Brexanolone aligns well with the journal scope. The study emphasizes rapid, precise, accurate, and stability-indicating analytical methodology according to ICH guidelines, which is highly relevant to the pharmaceutical and analytical research community targeted by IJDDT. Additionally, the journal provides a suitable platform for dissemination of applied pharmaceutical analytical research with industrial significance.

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