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FORCED DEGRADATION STUDIES OF BELZUTIFAN BY RP- HPLC

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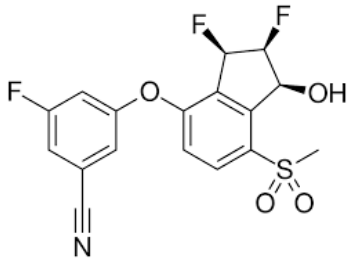
ABSTRACT
A strong and effective method for analyzing Belzutifan was put together. To achieve separation, we used an Inertsil column (150 mm × 4.6 mm, 5 µm) and a mobile phase made up of 0.1% orthophosphoric acid and acetonitrile, mixed with water in 60:40 ratio. We kept the flow rate at 1 mL/min and detected the UV at 303 nm. The column temperature was set at 30°C, and we used the mobile phase as the diluent. Our studies on degradation showed that the purity level was always higher than the purity angle, which indicates that the method is stable and trustworthy. While we didn't carry out a complete validation, the results hint that this method could work well for the routine analysis of Belzutifan in pharmaceutical products once it is validated.

KEYWORDS: HPLC Belzutifan, Method development. ICH Guidelines.

INTRODUCTION
Stability testing is an essential part of drug development, helping to determine how long a drug remains safe and effective under various conditions. For belzutifan, these tests follow the ICH Q1A guidelines, which outline the factors that can affect drug stability and help design methods to detect degradation. According to these guidelines, the stability of belzutifan depends on its chemical structure and how the drug is formulated. Forced degradation studies of Belzutifan using RP-HPLC help us understand how the drug breaks down under stress, like heat, light, or acid. RP-HPLC separates and measures these breakdown products, ensuring the drug stays safe and effective. This is crucial for meeting regulatory standards and understanding how the drug behaves over time. Belzutifan is an inhibitor of hypoxia-inducible factor 2α used as an antineoplastic in the treatment of certain cancers associated with von Hippel-Lindau (VHL) disease. Belzutifan is indicated for the treatment of adult patients with von Hippel-Lindau (VHL) disease who require therapy for associated renal cell carcinoma (RCC), central nervous system (CNS) hemangioblastomas, or pancreatic neuroendocrine tumors (pNET), who do not require immediate surgery. Belzutifan exerts its therapeutic effects by inhibiting a transcription factor necessary for the growth of solid tumors associated with VHL disease. It is taken once daily at approximately the same time each day, with or without food. Both severe anemia and hypoxia have been observed following therapy with belzutifan, and patients should be monitored closely before and during therapy to ensure patients can be managed as clinically indicated. There are no data regarding the use of erythropoiesis-stimulating agents for the treatment of belzutifan-induced anemia, and as such these therapies should be avoided. Hypoxia-inducible factor 2α (HIF-2α) is a transcription factor which aids in oxygen sensing by regulating genes that promote adaptation to hypoxia. In healthy patients, when oxygen levels are normal, HIF-2α is broken down via ubiquitin-proteasomal degradation by von-Hippel Lindau (VHL) proteins. In the presence of hypoxia, HIF-2α translocates into cell nuclei and forms a transcriptional complex with hypoxia-inducible factor 1β (HIF-1β) - this complex then induces the expression of downstream genes associated with cellular proliferation and angiogenesis. In patients with VHL disease-associated renal cell carcinoma, the mean C_{max} and AUC0-24h at steady-state - which was achieved after approximately three days of therapy - were 1.3 µg/mL and 16.7 µg•hr/mL, respectively.1 The median T_{max} is one to two hours following oral administration. Volume of distribution is 130L. Protein Binding is 45%. Metabolism: Belzutifan is primarily metabolized by UGT2B17 and CYP2C19, and to a lesser extent by CYP3A4. Half-life of belzutifan 14 hrs. Clearance rate 7.3 L/h. Brand name: Welireg.

MATERIALS AND METHODS
DRUG PROFILE
Belzutifan is an inhibitor of hypoxia-inducible factor 2α used as an antineoplastic in the treatment of certain cancers associated with von Hippel-Lindau (VHL) disease.

Structure



CAS Number : 1672668-24-4
IUPAC Name : 3-{[(1S,2S,3R)-2,3-difluoro-1-hydroxy-7-methanesulfonyl-2,3-dihydro-1H-inden-4-yl]oxy}-5-fluorobenzonitrile
Molecular Weight : Average: 383.34
Monoisotopic : 383.043913533
Molecular Formula: $C_{17}H_{12}F_3NO_4S$
Appearance : Powder
Physical State : Solid
Solubility : water soluble (0.0794 mg/mL)
Log p : 1.88
pK Values : pKa: 12.53 (Acidic), -3.7 (Basic).

EQUIPMENTS AND CHEMICALS

- **Materials:** Belzutifan pure drugs (API), Combination Belzutifan cream 1% (Welireg), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Equipment and Apparatus used

1. HPLC instrument used was of WATERS HPLC 2965 SYSTEM with Auto Injector and PDA Detector. Software used is Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance for Belzutifan solutions.
2. Sonicator (Ultrasonic sonicator)
3. P^H meter (Thermo scientific)
4. Micro balance (Sartorius)
5. Vacuum filter pump

Reagents used

1. Methanol HPLC Grade (RANKEM)
2. Acetonitrile HPLC Grade (RANKEM)
3. HPLC grade Water (RANKEM)
4. Glacial Acetic acid

Methods

Buffer

500 ml of HPLC grade Acetonitrile was taken in a 1000ml of volumetric flask add about 500ml of milli-Q water added and degas to sonicate it for 10min.

LABEL CLAIM: 1% cream (total weight 30gms)
Brand name: Welireg

Methods development

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile &water taken in the ratio of 60:40.

Preparation of Standard stock solutions: Accurately weighed 10mg of Belzutifan is 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. (200µg/ml of Belzutifan)

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. (20µg/ml of Belzutifan).

Preparation of Sample stock solutions: 10 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with dibath for 15 min and promptly centrifuged. The supernatant layer was taken and methanol layer was collected.

Preparation of Sample working solutions (100% solution): 0.5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (20µg/ml of Belzutifan).

Preparation of buffer

0.1% OPABuffer: 1ml of Conc Ortho Phosphoric acid was diluted to 1000ml with water.

Degradation procedure

Oxidation

To 1 ml of stock solution of Belzutifan 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain (20ppm) solution and 10µl 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 Belzutifan 1 ml of 2N Hydrochloric acid was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain (20ppm) solution and 10µl 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 Belzutifan 1 ml of 2 N sodium hydroxide was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain (20ppm) solution and 10µl 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 105^0c for 6h to study dry heat degradation For HPLC study, the resultant solution was diluted to (20ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the (400ppm) solution to UV Light by keeping the beaker in UV Chamber for 7days or 200Watt hours/m^2 in photo stability chamber, the resultant solution was diluted to obtain (20ppm) solutions and 10µl 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 HPLC study, the resultant solution was diluted to (20ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

RESULTS AND DISCUSSIONS

Based on drug solubility and Pk_a Value following conditions has been used to develop the method estimation of Belzutifan.

Method development: Method development was done by changing various, mobile phase ratios, buffers and columns etc.

Trial 1

Chromatographic co6nditions

Mobile phase : Water and Methanol taken in the ratio 60:40
Flow rate: 1ml/min
Column : Kromasil C18 (4.6 x 250mm, 5µm)
Detector wave length: 303.0nm
Column temperature: 30°C
Injection volume : 10µL
Run time : 10 min
Diluent : Water and Acetonitrile in the ratio 60:40
Results : Peak having tailing, so further trail was carried out.

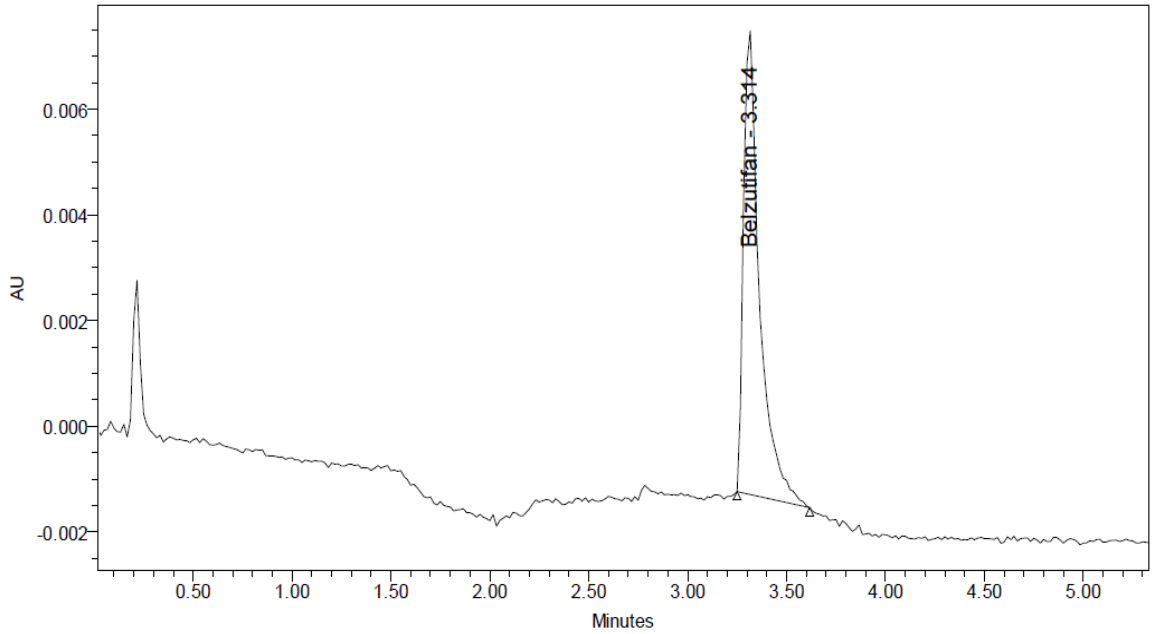


Fig. 1: Trial Chromatogram 1.

Trial 2

Chromatographic conditions

Mobile phase: Water: Acetonitrile (60:40)
Flow rate: 1ml/min
Column : Kromasil C18 (4.6 x 250mm, 5µm)
Detector wave length: 303 nm
Column temperature: 30°C
Injection volume: 10µL
Run time: 10.0 min
Diluent: Water and Acetonitrile in the ratio (60:40)
Results: In this trail also Peak having tailing so, further trial is carried out.

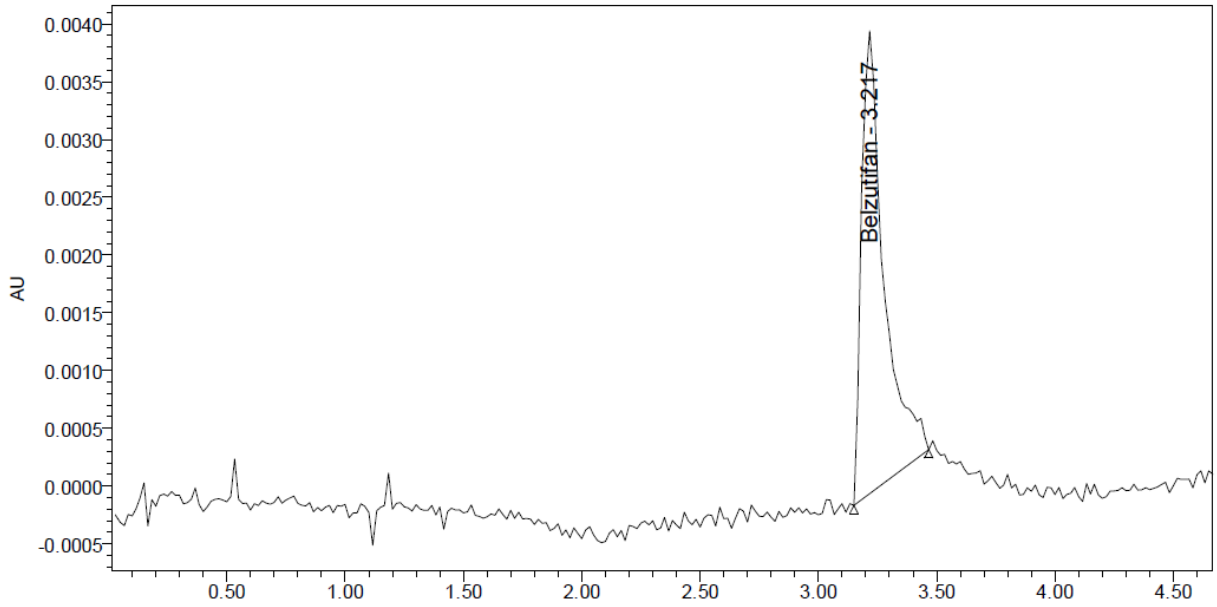


Fig. 2: Trial chromatogram 2.

Trial 3
Chromatographic conditions
Mobile phase: Acetonitrile: 0.01N kh2po4 (60:40)
Flow rate: 1ml/min
Column : Kromasil C18 (4.6 x 250mm, 5µm)
Detector wave length: 303.0nm
Column temperature: 30°C
Injection volume: 10µL
Run time: 6 min
Diluent: Water and Acetonitrile in the ratio 60:40
Results: By changing the solvent, peak shape was not good so, further trail was carried out.

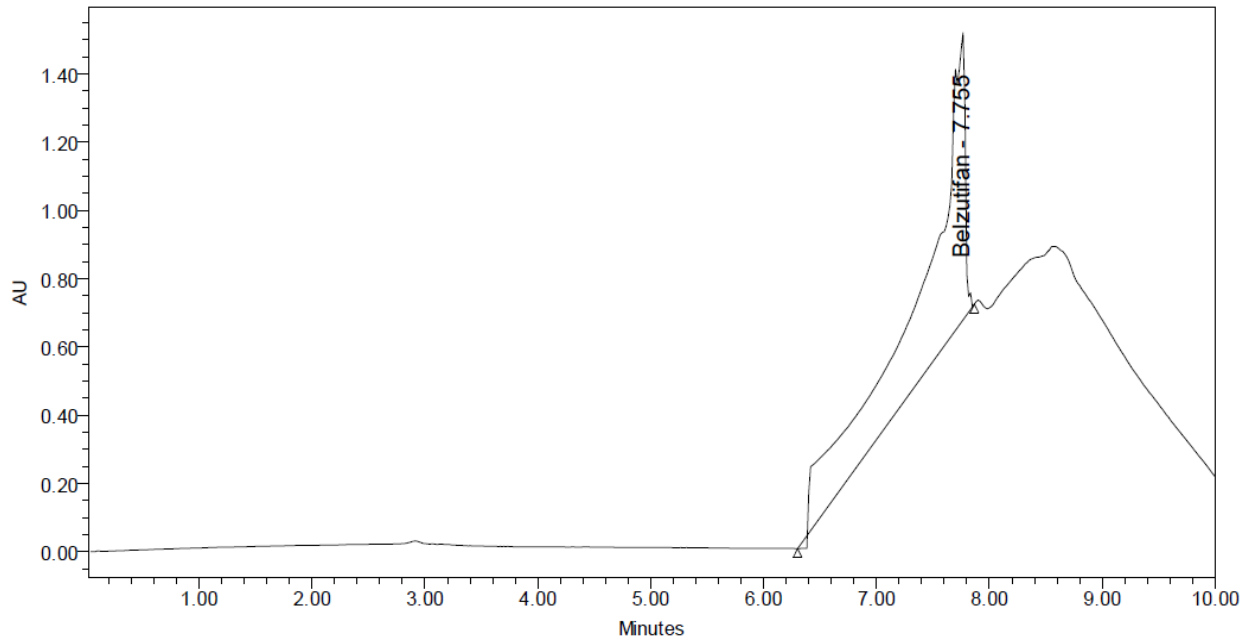


Fig. 3: Trial chromatogram 3.

Trial 4
Chromatographic conditions
Mobile phase: Acetonitrile: 0.1% OPA (60:40)
Flow rate: 1 ml/min
Column : Kromasil C18 (4.6 x 250mm, 5µm)
Detector wave length: 303.0nm
Column temperature: 30°C
Injection volume: 10µL
Run time: 10 min
Diluent: Water and Acetonitrile in the ratio 60:40
Results: Broad Peak shape observed so further trail was carried out.

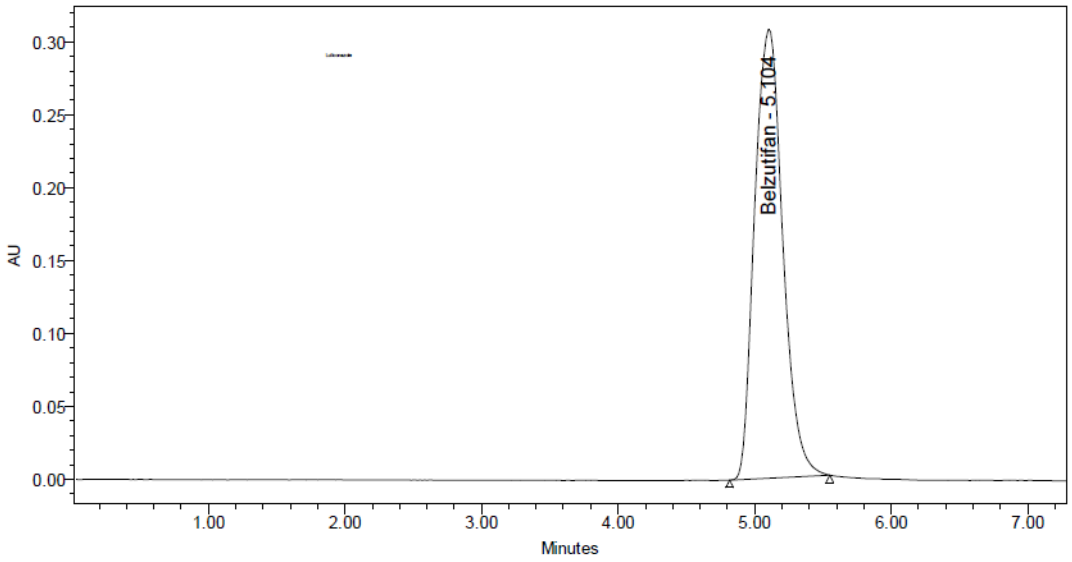


Fig. 4: Trial chromatogram 4.

Optimized conditions
Chromatographic conditions
Mobile phase: 0.1% OPA: Acetonitrile (60:40v/v)(2.2ph)
Flow rate: 1 ml/min
Column : Inertsil C18 (4.6 x 150mm, 5µm)
Detector wave length: 303.0nm
Column temperature: 30°C
Injection volume : 10µL
Run time: 10 min
Diluent: Water and Methanol in the ratio 60:40
Results: In this trail Chromatographic Conditions All the system suitability parameters are within the Limit and satisfactory. So this method was optimized.

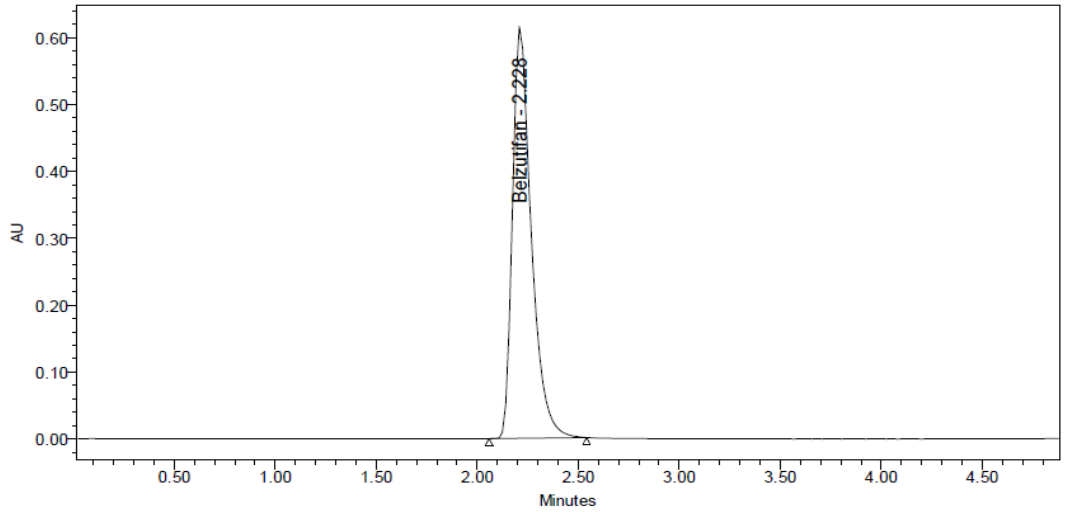


Fig. 5: Optimized chromatogram.

Observation: Belzutifan were eluted at 2.228 minutes respectively with good peak shape. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

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 6.8: Degradation Data of Belzutifan.

S. No	Degradation Condition	Peak area	% Drug un Degraded	% Drug Degraded
1	Acid	3403468	95.70	4.30
2	Alkali	3405804	95.76	4.24

3	Oxidation	3382397	95.10	4.90
4	Thermal	3504088	98.53	1.47
5	UV	3512390	98.76	1.24
6	Water	3544116	98.76	1.24

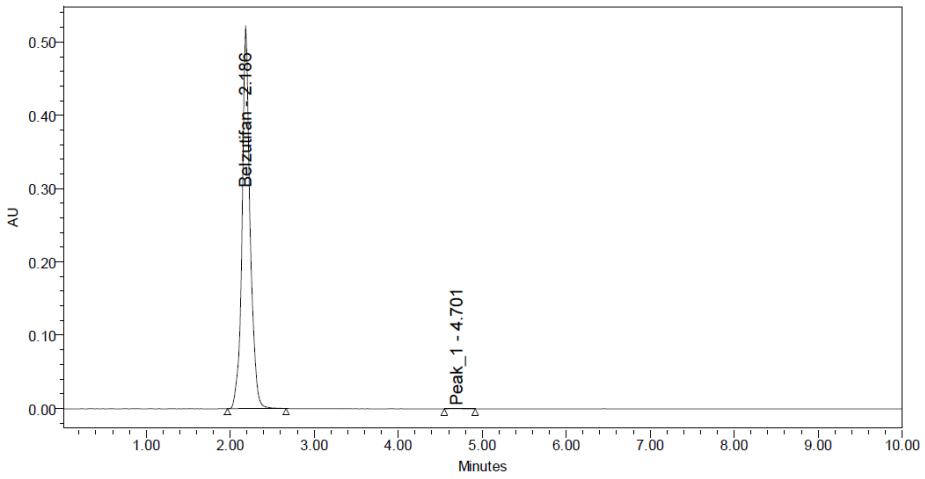


Fig. 6: Acid degradation chromatogram.

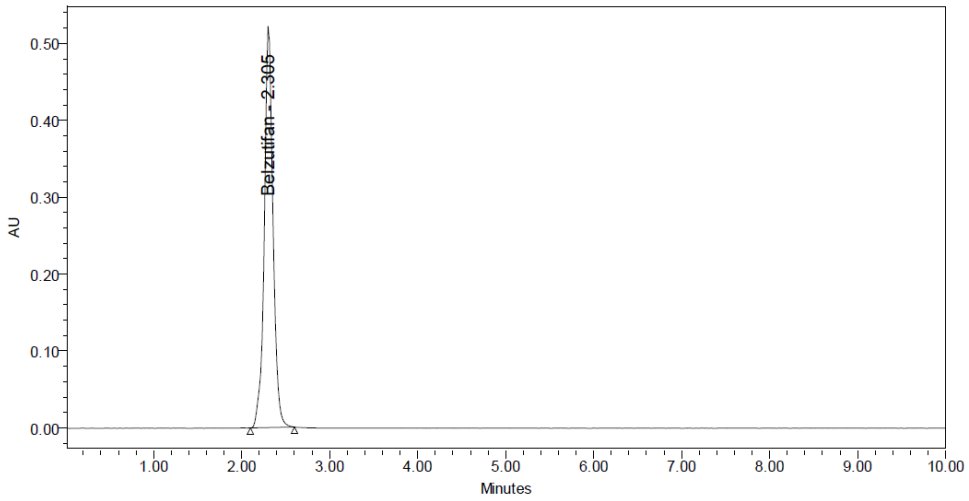


Fig. 7: Base degradation chromatogram.

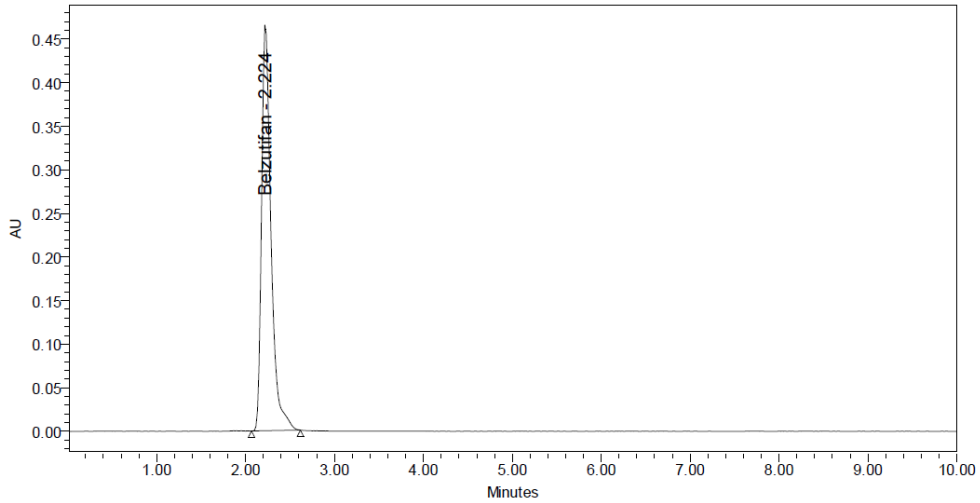


Fig. 8: Peroxide degradation chromatogram.

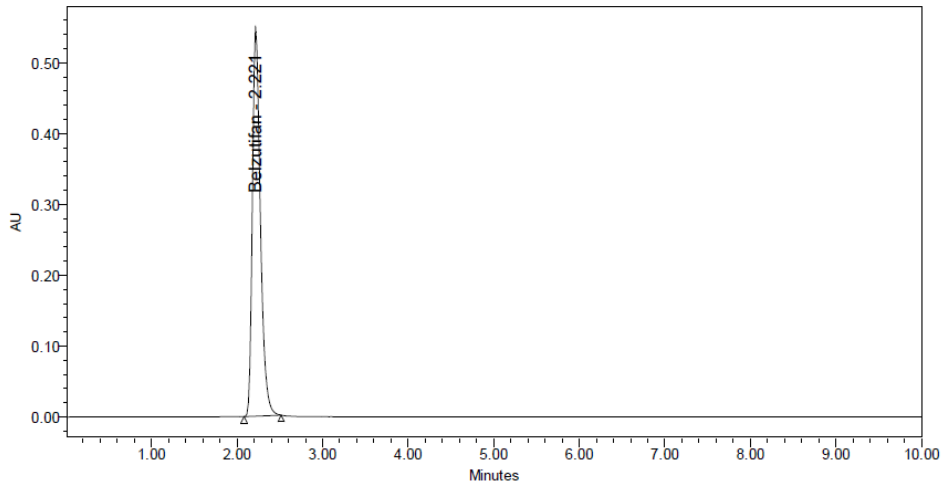


Fig. 9: Thermal degradation chromatogram.

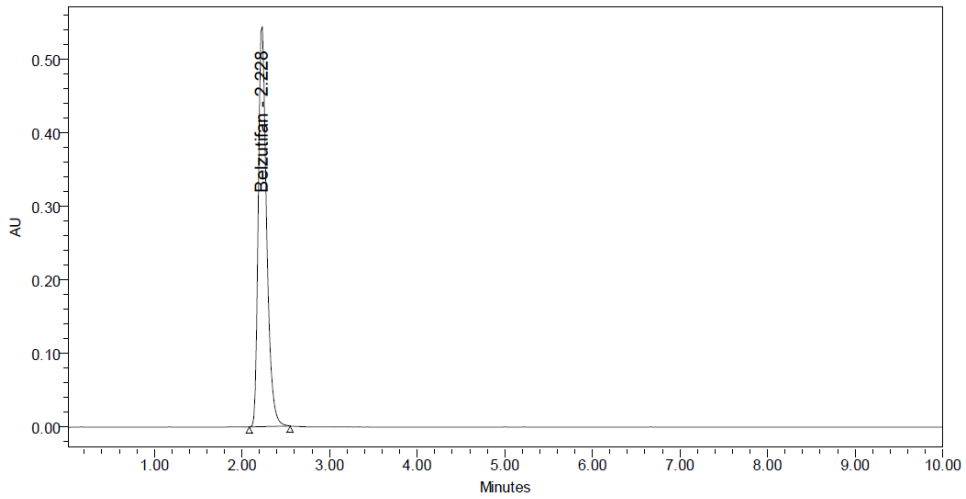


Fig. 10: UV degradation chromatogram.

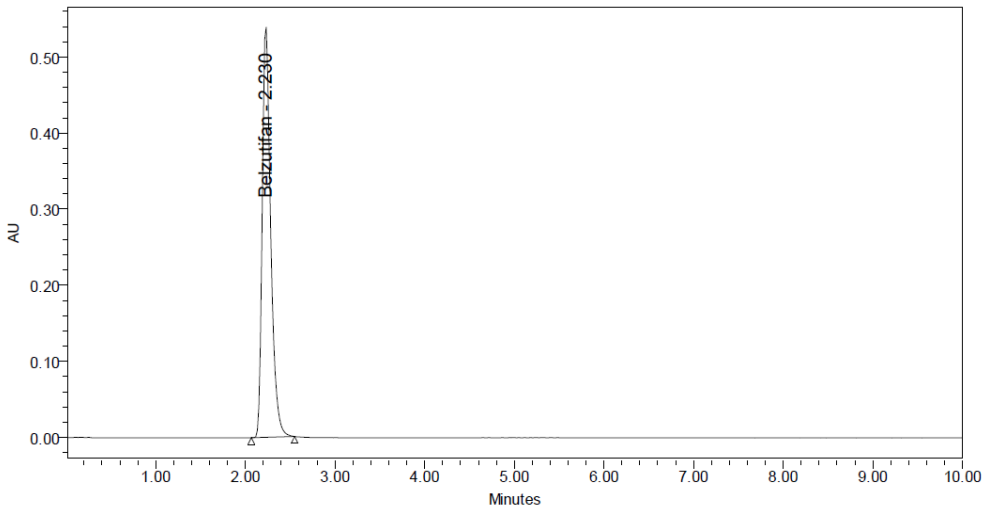


Fig. 11: Water degradation chromatogram.

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.

CONCLUSION

Chromatographic conditions used are stationary phase Inertsil 150mm x 4.6 mm, 5µm, Mobile phase 0.1% OPA: Acetonitrile (60:40) and flow rate was maintained at 1ml/min, detection wave length was 303nm, column temperature was set to 30^{o}C and diluent was mobile phase Conditions were finalized as optimized method. Degradation studies of Belzutifan were done, in all conditions purity threshold was more than purity angle and within the acceptable range. Full length method was not performed; if it is done this method can be used for routine analysis of Belzutifan.

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