Development of a stress induced validated UPLC-PDA method for the analysis of Eslicarbazepine acetate

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Abstract
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1. Introduction
Chemical stability of the pharmaceutical molecule is one of the most important concerns as it impacts the quality and safety of a drug. To discern the drug stability with various environmental factors, regulatory guidelines stipulate the requirement of stability testing data of drug substance and drug product (Wang et al., 1997; Blessy et al., 2014). According to the International Conference on Harmonization (ICH), this stability testing data assist the probable degradation products and generate the degradation pathways. Furthermore, it also facilitates the study of the molecule intrinsic stability and validation of proposed analytical method which enables the quantification of a drug in existence of degradation products. ICH Q1A (R2) suggested stability indicating analytical method which is suitably validated should be considered for stability studies. ICH Q2A, Q2B, and Q2C, and FDA 21 CFR section 211 recommend the development and validation of stress induced assay (ICH, 2003; ICH, 2005; Singh et al., 2000).

Ultra Performance Liquid Chromatography (UPLC) found to be a more auspicious chromatographic technique used for stress induced studies. It provides rapid chromatographic separation, high analysis, great resolution and good sensitivity. UPLC is a distinctly unique approach in liquid chromatography in term of smaller size particle, optimization of system, model of detector and refinement of data (Novakova et al., 2006; Swartz, 2005). Solvent manager, sample manager, column, column heater and detectors are the five main components of UPLC. The pulse free mobile phase flow at an analytical flow rate through the system is managed by the solvent manager. Further, sample manager injects the acquired samples from vials into the chromatographic flow stream. The particle size less than 2 μm provide good resolution, speed and sensitivity. Widespread preference is ACQUITY UPLC BEH C18 column, as it provides widest pH range. The ACQUITY UPLC BEH C18 column packed with 1.7-μm, bridged, ethylsiloxane, hybrid particles bear high pressure conditions. It provides good retention of the compound by high optimum velocity and low plate height. For better detection of an analyte, the photodiode array (PDA) detector provides highly sensitive spectral information. PDA operating range is 190 and 500 nm; it is based on ultraviolet or visible light source (Taleuzzaman et al., 2015; Mazzeo et al., 2005).

Stress degradation studies provide an approach to analyse the stability of drug samples. Stability information of molecule provides the data to select proper formulation, package, storage conditions and shelf life. These data also play a significant role which is required in regulatory documentation. Eslicarbazepine acetate (ESA) is a pro-drug. ESA after administration converted to eslicarbazepine (S-licarbazepine). It is an active metabolite of oxcarbazepine (Almeida et al., 2007; Alves et al., 2007; Alves et al., 2010). The modification in structure not only improved efficacy, safety but also avoid the toxic epoxide metabolite formation in ESA. Chemically ESA is (S)-(-)-10-acetoxy-10,11-dihydro-5H-dibenz [b, f] azepine-5-carboxamide (Fig. 1) (https://en.wikipedia.org/wiki/Eslicarbazepine_acetate). ESA is a potent

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Eslicarbazepine acetate (ESA) was procured gratuitously by Arbro Pharmaceuticals Pvt. Ltd. Analytical grade chemicals and materials were used throughout the study. Hydrochloric acid, hydrogen peroxide (30%), sodium hydroxide was procured from SD Fine-chem. Acetonitrile (ACN), methanol (MeOH) from Merck India (Mumbai). All the reagents and solutions were made from HPLC grade water from Merck India (Mumbai).

2.2. Instrumentation

The purity of ESA (>98% pure) was checked by using UPLC-MS/MS. Sample analysis was carried on UPLC Waters Acquity system (Waters Corporation, Milford, Massachusetts, USA) assembled through the binary solvent manager, sample manager (auto sampler) and a PDA detector using Waters Acquity BEH 150 × 2.1 mm, 1.7 μm, C18 column for chromatographic separation in stress degradation studies. Achieved signal was acquired and processed via Empower software. The separation was accomplished by means of mobile phase A and mobile phase B in the proportion of 50:50 (V/V) at 0.2 mL/min flow rate. Mobile phase A comprises a mixture of 0.01 M potassium dihydrogen orthophosphate and acetonitrile (90:10, V/V) and mobile phase B comprises a mixture of acetonitrile-water-methanol (75:5:25, V/V/V). Methanol was used as a diluent. The injection amount was 2 μL, column temperature was 30 °C and the eluent was identified at the wavelength of 215 nm. Millipore filter assembly with 0.45 μm nylon membrane was used for the filtration of mobile phase and microprocessor water proof pH tester (pH tester 20, Eutech Instruments, Oakton, USA) was used to adjust the pH of the mobile phase. Hot air oven (Oven universal with thermotech thermostat TIC-4000 N, S.M. Industries, New Delhi, India) was used for thermal studies. For hydrolytic degradation studies, the drug was refluxed in a round bottom flask condenser assembly.

2.3. Preparation of standard stock solution

Accurately weigh 10 mg of ESA and transfer into a 10 mL volumetric flask and conjured to volume with methanol to give a stock solution of 1 mg/mL. Furthermore, by diluting the stock solution with a diluent, the standard solution of desired concentration was made in stress degradation studies.

2.4. Stress induced degradation studies

To establish the stability indicating property of the drug, stress degradation studies were executed. As per ICH Q1A (R2) stress degradation studies were executed under conditions of acidic hydrolysis, alkaline hydrolysis, neutral hydrolysis, oxidation, dry heat and photolysis.

According to the ICH guidelines ascending order concentration of acid, base and peroxide should be selected, like start with 0.001 N to highest concentration till significant degradation is achieved. For each stress condition four samples were made viz., blank solution (stored in ambient condition), stressed blank solution (subjected to stress conditions), drug solution (Eslicarbazepine acetate stored in ambient condition), stressed drug solution (Eslicarbazepine acetate subjected to stress conditions).

2.4.1. Acid hydrolysis

For acid hydrolytic stress degradation studies, 1 mL of stock solution and 1 mL of 2 N HCl in 10 mL volumetric flask were taken and volume was conjured to 10 mL with methanol. The solution was refluxed at 60 °C for 15 min. The solution was allowed to cool at room temperature and neutralized with NaOH to 7 pH. Final solution was analyzed for degradation studies.

2.4.2. Alkaline hydrolysis

Alkaline hydrolytic stress degradation studies were performed by taking 1 mL of stock solution and 1 mL of 0.001 N NaOH in 10 mL volumetric flask. Further, the volume was conjured to 10 mL with methanol and the solution was refluxed at 60 °C for 15 min. The solution was allowed to cool at room temperature and neutralized with HCl to 7 pH. Final solution was analyzed for degradation studies.

2.4.3. Neutral hydrolysis

1 mL of stock solution with 1 mL of HPLC grade water in 10 mL of volumetric flask was taken. Further the volume was conjured to 10 mL with methanol and the solution was refluxed at 60 °C for 48 h. The solution was allowed to cool at room temperature and analyzed for degradation studies.

2.4.4. Oxidative stress

For oxidative stress degradation studies 1 mL of stock solution and 1 mL of 30% H₂O₂ was taken in 10 mL volumetric flask and volume was conjured to 10 mL with methanol. Further the solution was refluxed at 60 °C for 24 h. The solution was allowed to cool at room temperature and analyzed for degradation studies.

2.4.5. Thermal stress

For thermal stress degradation studies solid sample of drug was bared to dry heat condition at 100 °C for 10 days. Further the drug is diluted with a diluent and final solution was analyzed for degradation studies.

2.4.6. Photolytic stress

For photolytic studies the dry powder of drug were bared to sunlight during the daytime (60,000–70,000 lux) for 48 h. Further the drug is diluted with a diluent and final solution was analyzed for degradation studies.
2.5. Method development

For analysis of stress degradation studies and characterization of degradation product Waters Acquity UPLC system (Waters Corporation, Milford, Massachusetts, USA) was used. The system was assembled with a binary solvent manager, sample manager (auto sampler) and a PDA detector. Literature survey enlightens a few HPLC methods for a stress degradation study of ESA. The developed methods were either on C8 or C18 columns with altered column temperature, mobile phase and pH. The UPLC method with rapid chromatographical separation, high analysis, excellent resolution and good sensitivity was developed by logical modification in column, column temperature, mobile phase composition, pH and flow rate. Hence, Waters Acquity BEH 150 × 2.1 mm, 1.7 μm, C18 column for chromatographic separation in stress degradation studies at 30 °C column temperature with 0.01 M potassium dihydrogen orthophosphate and acetonitrile (90:10, V/V) as mobile phase A and acetonitrile-water-methanol (75:5:25, V/V/V) as mobile phase B in the proportion of 50:50 was used respectively. Mobile phase was filtered with Millipore filter assembly with 0.45 μm nylon membrane prior to use. 0.2 mL/min flow rate with 2 μL injection volume at 215 nm wavelength was used. Data was analyzed on Empower software. Firstly, stress degradation studies on UPLC were carried out on all reaction solutions separately. Further, stress degradation studies were executed on a mixture of degraded drug solutions.

2.6. Method validation

The UPLC developed method was validated as per ICH guideline Q2 (R1) in term of various parameters:

### 2.6.1. Linearity and range

A stock solution 1 mg/mL drug in methanol was diluted to get the solution in the concentration range of 10–50 μg/mL. In triplicate the solutions were injected into the UPLC column, keeping the 2 μL the amount of injection constant.

### 2.6.2. Precision

To examine the precision of the developed method six injections of five different concentrations (10–50 μg/mL) were prepared on the similar day of study and relative standard deviation (% RSD) were calculated to verify the intra-day precision. For the inter-day precision evaluation these studies were carried out on different days.

### 2.6.3. Accuracy

For the accuracy evaluation recovery studies were carried out. The decomposed reaction solutions mixture was fortified with five known concentrations of this drug; further recovery of added drug was estimated.

### 2.6.4. Specificity and selectivity

The ability of the analytical method to measure the response of the analyte in the existence of obstructive substances including impurities and its degradation products is specificity. The specificity was evaluated by resolution factor of the drug peak from the nearby peak. The purity of each degradation peak will specify the selectivity of the method.

### 2.6.5. Robustness

The evaluation of robustness of the method was carried out by the study of effect of small modification of method parameters such as flow rate (±10%), column temperature (±5 °C), mobile phase (±2%), and wavelength of detection (±5%) and pH of the buffer in the mobile phase (±0.2%). In this evaluation only one parameter is modified and the remaining parameters were kept constant.

### 2.6.6. Limit of detection (LOD) and limit of quantitation (LOQ)

The lowest level of analyte that can be detected and gives a measurable response is termed as LOD, whereas the smallest amount of analyte that can be reproducibly quantified is termed as LOQ. LOD and LOQ were evaluated by injecting decreasing con-

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**Table 1**

| PEAKS             | RT    | RRT |
|-------------------|-------|-----|
| DP                | 1.83  | 0.695 |
| Eslicarbazepine Acetate | 2.63  | 1.000 |

Rt: Retention time; RRT: Relative retention time.

**Fig. 2.** Representative chromatogram of eslicarbazepine acetate (ESA) [Conditions: Waters Acquity BEH C18 column (150 × 2.1 mm, 1.7 μm); mobile phase A (mixture of 0.01 M potassium dihydrogen orthophosphate and acetonitrile (90:10, V/V)) and mobile phase B (mixture of acetonitrile-water-methanol (75:5:25, V/V/V)) in the proportion of 50:50 (V/V) at 0.2 mL/min flow rate; UV-detection at 215 nm at temperature (30 ± 1 °C)]. The chromatogram of ESA showing retention time 2.63 min.
centration of drug by the following formula, where SD is the standard deviation of the response and S is the slope of the calibration plot.

LOD = \frac{3.3}{S} \times SD \quad \text{and} \quad \text{LOQ} = \frac{10}{S} \times SD

3. Results and discussion

3.1. Stress induced analysis of ESA

UPLC studies on ESA under diverse stress conditions revealed that ESA does not degraded in acid hydrolysis, neutral hydrolysis, oxidative, thermal and photolytic stresses. However, ESA showed prominent degradation in alkaline hydrolytic condition. Alkaline hydrolytic condition showed one distinct degradation product in a mixture of a solution. Table 1 demonstrate the retention time 2.63 min (1.000 RRT) along with its degradant (DP 1) at 1.83 min (0.695 RRT).

![Representative chromatogram of eslicarbazepine acetate (ESA) and its degradation product (DP 1) in a mixture of stressed alkaline solution](image)

The chromatogram of ESA showing retention time 2.63 min (1.000 RRT) along with its degradant (DP 1) at 1.83 min (0.695 RRT).

Fig. 3. Representative chromatogram of eslicarbazepine acetate (ESA) and its degradation product (DP 1) in a mixture of stressed alkaline solution [Conditions: Waters Aquity BEH C18 column (150 x 2.1 mm, 1.7 µm); mobile phase A (mixture of 0.01 M potassium dihydrogen orthophosphate and acetonitrile (90:10, V/V)) and mobile phase B (mixture of acetonitrile-water-methanol (75:5:25, V/V/V)) in the proportion of 50:50 (V/V) at 0.2 mL/min flow rate; UV-detection at 215 nm at temperature (30 ± 1 °C)]. The chromatogram of ESA showing retention time 2.63 min (1.000 RRT) along with its degradant (DP 1) at 1.83 min (0.695 RRT).

The alkaline hydrolysis of amide result into acid so alkaline degradation product was observed, which can be understand by following mechanism:

![Chemical structures showing the degradation mechanisms of eslicarbazepine acetate](image)
The drug demonstrates stability in other stress conditions. In acidic stress condition it was found that at 60 °C in 5 N HCl for 24 h of reflux no degradation occurred. In neutral hydrolysis when drug was refluxed for 5 days at 60 °C in water the drug was observed to be stable. In oxidative stress condition the drug was again exhibit stability when refluxed for 5 days in 30% H2O2 at 60 °C. Degradation observation was none when drug was exposed to sunlight (60,000–70,000 lux) for 48 h and at 100 °C in oven for 30 days signifying that the drug showed stability in photolytic and thermal stress conditions respectively. The RT, RRT and resolution of peaks in the chromatograms confirmed the developed UPLC method was specific and selective.

3.2. Method development and optimization

Thus stability indicating ability of ESA was established using UPLC and it was suggested that the drug showed significant degradation in alkaline hydrolytic condition. Primarily, stress degradation studies was carried on C18 BEH (150 × 2.1 mm, 1.7 μm) column at 30 °C using different proportion of acetonitrile and water as mobile phase, but good resolution was not achieved. Afterwards potassium dihydrogen orthophosphate with water as buffer was used, but peak shape was not acceptable. However, replacing water with acetonitrile not only decreased the retention time but also gives good sharp peak. Thus potassium dihydrogen orthophosphate with acetonitrile was made as a choice of buffer. Consequently trials were made by means of altered proportions of acetonitrile, water and methanol. However, best separation with good resolution and peak shape was achieved using a mixture of acetonitrile-water-methanol in the proportion of 75:5:25, V/V/V. To achieve complete separation and good resolution, the chosen ratio of buffer and mixture of solution was 50:50 (% V/V). Instantaneously PDA detector was monitored at constant 0.2 mL/min flow rate, at the wavelength of 215 nm using C18 BEH (150 × 2.1 mm, 1.7 μm) column and 30 °C column temperature.

3.3. Method validation

The developed UPLC method was successfully validated in reference of ICH guideline Q2 (R1). The developed method was satisfactorily specific for ESA (Fig. 2). Fig. 3 showed that the method stays selective for all components in a mixture of stressed solutions. Table 1 shows the RT and RRT of ESA and its degradation product signifying the selectivity of the method. The LOD and LOQ were found to be 0.382 μg/mL and 1.15 μg/mL, respectively. The linearity was checked by linear regression analysis. It was found that the response of the drug was strictly linear in 10–50 μg/mL concentration range (Fig. 4). The linearity results are given in Table 2. The equation of regression analysis obtained for ESA: Y = 5098.2X + 2626.8. The correlation coefficient was observed to be R² = 0.999. Table 3 exemplified the data obtained from intra-day and inter-day precision studies. The results signifies that the method was sufficiently precise as the %RSD values for the precision studies was found to be <1%. At the various added concentrations excellent recoveries of the drug was obtained in the range

![Fig. 4. Linearity graph of eslicarbazepine acetate (ESA).](image-url)

**Table 2**

Linearity data for eslicarbazepine acetate.

| Concentration (μg/ml) | AUC ±SD | RSD (%) |
|-----------------------|---------|---------|
| 10                    | 53453.00| 0.099   |
| 20                    | 105132.83| 0.067   |
| 30                    | 154759.00| 0.336   |
| 40                    | 207171.00| 0.294   |
| 50                    | 257341.67| 0.159   |

AUC: Area under the curve; SD: standard deviation; RSD: relative standard deviation.

**Table 3**

Precision data for eslicarbazepine acetate (n = 6).

| Actual concentration (μg/ml) | Intra-day | Inter-day |
|-----------------------------|-----------|-----------|
|                             | Measured concentration ±SD | RSD (%) | Measured concentration ±SD | RSD (%) |
| 10                          | 09.958 ±0.045 | 0.452 | 09.817 ±0.094 | 0.955 |
| 20                          | 20.016 ±0.169 | 0.847 | 20.086 ±0.075 | 0.375 |
| 30                          | 30.126 ±0.201 | 0.667 | 30.053 ±0.245 | 0.814 |
| 40                          | 40.210 ±0.221 | 0.549 | 40.131 ±0.086 | 0.213 |
| 50                          | 50.039 ±0.330 | 0.660 | 50.019 ±0.124 | 0.247 |

SD: standard deviation; RSD: relative standard deviation.

**Table 4**

Recovery data for eslicarbazepine acetate (n = 4).

| Concentration (μg/ml) | Calculated spiked concentration | ±SD | RSD (%) | Recovery (%) |
|-----------------------|---------------------------------|-----|---------|--------------|
| 10                    | 09.915 ±0.053 | 0.529 | 99.153 |
| 20                    | 20.113 ±0.098 | 0.487 | 100.566 |
| 30                    | 30.144 ±0.225 | 0.748 | 100.483 |
| 40                    | 39.984 ±0.243 | 0.608 | 99.960 |
| 50                    | 50.102 ±0.104 | 0.207 | 100.204 |

SD: standard deviation; RSD: relative standard deviation.
from 99.153% to 100.566% which has been illustrated in Table 4. By small alteration of mobile phase, the developed method was observed to be robust. Mobile phase A is 0.01 M potassium dihydrogen orthophosphate and acetonitrile (90:10, V/V) and mobile phase B consisted a mixture of acetonitrile-water-methanol (75:5:25, V/V). The data has been illustrated in Table 5.

4. Conclusion

As per ICH requirement, the stability indicating UPLC method for ESA was developed successfully. The developed method was found to be more acceptable as compared to conventional HPLC methods in term of rapid chromatographic separation, high analysis, excellent resolution and good sensitivity. The ICH guidelines have been followed during method validation and stress testing. Stress degradation studies demonstrate that ESA degrades in alkaline hydrolytic condition. One distinct degradation product appears in a mixture of a stress solution. While the drug show stability in acid hydrolysis, neutral hydrolysis, oxidative, thermal and photolytic stress conditions. All validation parameters give acceptable results in terms of specificity, selectivity, linearity, precision, accuracy and robustness. Thus the developed stability indicating method can be utilized for regular analysis to check the sample stability and to manufacture the drug and its combination pharmaceutical dosage forms.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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