INTRODUCTION

Escitalopram oxalate (ESC) is the (Figure 1) selective serotonin reuptake inhibitor, antidepressant agent, chemically it is S-(+)-1-[3-(dimethylamino) propyl]-1-(4-fluorophenyl)-1, 3-dihydro-2-benzofuran-5-carbonitrile [1,2].

Etizolam (ETI) (Figure 1) belongs to an original chemical class of diazepines, namely thienotriazolodiazepines with antianxiety activity and chemically it is 4-(2-Chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2-f][1,2,4] triazolo[4,3-a][1,4] diazepine 1H[3,4]. ESC is official in IP'10 and ETI is official in JP XV [3,4]. Literature survey indicate some spectrophotometric[5-11], HPLC [12-15], HPTLC [16-18], fluorimetry [19-20], LC-MS [21-24], LC-MS/MS [25], enantiomeric separation [26-27], CE [28] and TLC [29] methods for estimation of ESC either individually or in combination with other drugs. Literature survey also reports few HPLC [30], HPTLC [31], LC-MS [32, 33] and GC-MS [34, 35] methods for estimation of ETI individually or in combination with other drugs. However there is no analytical method reported for simultaneous estimation of both drugs in their combined tablet dosage form by reporting forced degradation studies to demonstrate stability indicating nature of the method. Present work describes rapid, simple, sensitive, accurate and reproducible stability indicating method. The present developed method was used determine the Escitalopram and Etizolam in their combined dosage forms.

MATERIALS AND METHODS

HPLC grade Ammonium Acetate, acetonitrile and water were procured from Merck India. All dilutions were performed in standard class-A, volumetric glassware. For the estimation of commercial formulation, Etizola plus having (Escitalopram oxalate-0.5mg and Etizolam-5mg) manufactured by Mecloeods pharmaceuticals pvt ltd were procured from the local market.

Instrumentation

Agilent 1120 compact LC chromatographic system, with DAD detector and a fixed injector equipped with 20µL loop was used for the chromatographic separation. The chromatogram was recorded at and peaks quantified by means of Ez Chrome software. Chromatographic separation was carried out on a C18 column [Inertsil ODS 3V, 150mm x 4.6mm 5µ]. Sartorious electronic balance was used for weighing the samples. Ultra-sonic bath sonicator was used for degassing and mixing of the mobile phase.

Chromatographic conditions

Chromatographic separation of Escitalopram and Etizolam was carried on a C18 column. The mobile phase composition of acetonitrile: methanol: 0.02M ammonium acetate buffer (30:20:50), v/v, pH 4.5) at a flow rate of 1.0 mL/min with UV detection at 227 nm. The retention time of Escitalopram and Etizolam were 2.3 min and 5.7 min respectively. The proposed method could be used for routine analysis of Escitalopram and Etizolam in their combined dosage forms.
phase was composed of acetonitrile, methanol and ammonium acetate buffer (pH 4.5) in the ratio of 30:20:50 v/v. It was filtered through a 0.45 µm membrane filter and degassed for 15 minutes. The flow rate of the mobile phase was maintained at 1 ml/min. Detection was carried out at 227 nm at ambient temperature.

**Method development**

**Preparation of Standard Stock Solutions:** Standard stock solutions were prepared by dissolving 25mg of Escitalopram and 50mg Etizolam working standard in two separate each 100 mL and 50mL volumetric flasks using 30mL of mobile phase and made up to the mark with mobile phase to obtain a final concentration of 250µg/mL and 1000µg/mL of each Escitalopram and Etizolam. From the above stock solutions, each 2ml and 5mL of aliquots of estitalopram and etizolam were pipette in to a 100mL volumetric flask and dissolved in 25mL of the mobile phase and made up to the mark with the solvent to obtain a final concentration of 5µg/mL and 50µg/mL for Escitalopram and Etizolam respectively.

**Preparation of Sample solutions:** Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 5mg Escitalopram and 50mg of Etizolam into a 100 mL volumetric flask, added 70 mL of diluent and sonicated for 30minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transferred 5.0mL of the above solution into a 100 mL volumetric flask and diluted to volume with diluent to obtain a concentration of 5 and 50µg/mL of Escitalopram and Etizolam respectively.

**Method validation:** The developed HPLC method for the simultaneous determination of Escitalopram and Etizolam was validated as per the ICH guidelines13, 14. As part of method validation as per ICH guidelines, the following parameters are studied. Each parameter was explained separately in different sections under results and discussions.

1. System Suitability and System Precision
2. Specificity Studies
   a) Blank Interference
   b) Placebo Interference
   c) Forced degradation studies in different stress conditions to establishing stability indication of the developed method.
3. Method Precision
4. Accuracy studies
5. Linearity Studies including LOD/LOQ determination
6. Ruggedness
7. Robustness
8. Analysis of Marketed samples by applying the developed method.

**RESULTS AND DISCUSSION**

**System suitability and System Precision**

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), Resolution, Tailing factor. The HPLC system was equilibrated using the initial mobile phase composition, followed by 5 injections of the standard solution of 100% concentration containing 5µg/mL Escitalopram and 50 µg/ml Etizolam. These 5 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the Table 1.

**Table 1:** System suitability parameters for Escitalopram and Etizolam by proposed method

<table>
<thead>
<tr>
<th>Name of the Compound</th>
<th>Retention Time</th>
<th>Tailing factor</th>
<th>Theoretical plate</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escitalopram</td>
<td>2.307</td>
<td>1.68</td>
<td>2761</td>
<td></td>
</tr>
<tr>
<td>Etizolam</td>
<td>5.740</td>
<td>1.54</td>
<td>4883</td>
<td>13.620</td>
</tr>
</tbody>
</table>

**Specificity**

**Blank interference:** A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the defined above chromatographic conditions and the blank chromatograms were recorded. Chromatogram of Blank solution (Figure 2) showed no peaks at the retention time of Escitalopram and Etizolam peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Escitalopram and Etizolam in Escitalopram and Etizolam tablets. Similarly typical representative chromatogram of standard is also shown (Figure 3).
Figure 3: A typical HPLC Chromatogram showing the peak of Escitalopram and Etizolam

**Forced Degradation:**

**Control Sample:** Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 5 mg Escitalopram and 50 mg of Etizolam into a 100 mL volumetric flask, added 70 mL of diluent, and sonicated for 30 minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transferred 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent (Figure 4A).

**Acid Degradation Sample:** Weigh and finely powdered not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 5 mg of Escitalopram and 50 mg of Etizolam into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 mL of 1N acid, refluxed for 30 min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent (Figure 4B).

**Base Degradation Sample:** Weigh and finely powdered not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 5 mg of Escitalopram and 50 mg of Etizolam into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 mL of 1N NaOH, refluxed for 30 min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent (Figure 4C).

**Peroxyde Degradation Sample:** Weigh and finely powdered not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 5 mg of Escitalopram and 50 mg of Etizolam into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature. Then add 5 mL of Hydrogen Peroxide, refluxed for 30 min at 60°C, then cooled to room temperature, and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent (Figure 4D).

**Thermal Degradation Sample:** Powder collected from 20 tablets is exposed to heat at 105°C for about 5 days. Accurately weigh and transfer equivalent to 5 mg of Escitalopram and 50 mg of Etizolam into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent (Figure 4E). Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by proposed method.
Figure 4C: A typical HPLC Chromatogram showing the profile of Escitalopram and Etizolam in Base hydrolysis by proposed method.

Figure 4D: A typical HPLC Chromatogram showing the profile of Escitalopram and Etizolam in Peroxide hydrolysis by proposed method.

Figure 4E: A typical HPLC Chromatogram showing the profile of Escitalopram and Etizolam in Thermal hydrolysis by proposed method.

**Linearity and range**

The standard curve was obtained in the concentration range of 3-7μg/ml for Escitalopram and 30-70 μg/mL for Etizolam. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient \([r^2]\) of standard curve were calculated and given in Figure-5A (For Escitalopram) and Figure 5B (For Etizolam) to demonstrate the linearity of the proposed method. The result of regression analysis was given in the Table 2. From the data obtained which given in Table 2 (For Escitalopram and Etizolam) the method was found to be linear within the proposed range.

**Table 2: Linearity studies for Escitalopram and Etizolam by proposed method**

| % Level (Approx.) | Escitalopram | | Etizolam | | |
|------------------|--|---|---|---|
|                  | Concentration (µg/ml) | Area | Concentration (µg/ml) | Area |
| 60               | 3 | 2002.3758 | 30 | 154.6592 |
| 80               | 4 | 2669.8344 | 40 | 219.5456 |
| 100              | 5 | 3337.2935 | 50 | 274.4323 |
| 120              | 6 | 3997.5461 | 60 | 329.3184 |
| 140              | 7 | 4572.2102 | 70 | 394.2048 |
|                  | Slope | 647 | | 6 |
|                  | Intercept | 82 | | 20 |
|                  | % Y-Intercept | 2.1 | | 6.1 |
|                  | STYEX | 34 | | 4 |
|                  | CC | 0.9996 | | 0.9994 |
|                  | RSQ | 0.9992 | | 0.9988 |
|                  | Residual sum of squares | 34 | | 4 |
|                  | LLD | 1.37 | | LLD |
|                  | LLQ | 4.15 | | LLQ |

**Figure 5A: Calibration curve for Escitalopram**

Linearity Curve for Assay of Escitalopram by Proposed Method

**Figure 5B: Calibration curve for Etizolam**

Linearity Curve for Assay of Etizolam by Proposed Method
Accuracy

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method. In the current study recovery at three spike levels 80%, 100% and 120% were carried out. The % recovery at each spike level was calculated and was given in Table 3.

<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
<th>Overall % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>99.2-99.8</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>99.9-100.5</td>
<td>0.30</td>
<td>0.78</td>
</tr>
<tr>
<td>120</td>
<td>98.5-101.2</td>
<td>1.37</td>
<td></td>
</tr>
</tbody>
</table>

Table 3B: Recovery studies for Etizolam by proposed method

<table>
<thead>
<tr>
<th>% Level</th>
<th>Recovery Range</th>
<th>% RSD at each level</th>
<th>Overall % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>98.2-99.9</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>98.4-100.1</td>
<td>0.99</td>
<td>0.83</td>
</tr>
<tr>
<td>120</td>
<td>99.5-100.8</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was considered at different levels, i.e. method, system, Inter day and intraday. Precision of the developed method was assessed by measuring the response on the same day (intraday precision) and next two consecutive days (inter day precision). The precision of the method was assessed by six replicate injections of 100% test concentration. Intra and inter-day precision of the method was assessed by determination of standard deviation and % RSD for the analyte response. The result was given in Table 4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>3.3 σ/S</td>
</tr>
<tr>
<td>LOQ</td>
<td>10 σ/S</td>
</tr>
</tbody>
</table>

LOD and LOQ

LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of the responses and S is the slope of the calibration curves). In the present method

Robustness

The robustness of the method was determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as percent organic content, pH of the mobile phase, buffer concentration, temperature, injection volume and flow rate. A deviation of ± 2nm in the detection wavelength, ± 0.1 mL/min in the flow rate, ± 5%change in the organic phase were tried individually. The result was given in the Table 5.

Table 5: Robustness studies for Escitalopram and Etizolam by proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>3.3 σ/S</td>
</tr>
<tr>
<td>LOQ</td>
<td>10 σ/S</td>
</tr>
</tbody>
</table>

Over all summary of the method

Column chemistry, solvent selectivity, solvent strength (volume fraction of organic solvent(s) in the mobile phase), detection wavelength and flow rate were varied to determine the chromatographic conditions for giving the best separation. Several mobile phase compositions were tried to resolve the peaks of Escitalopram and Etizolam. The optimum results were attained with acetonitrile, methanol and ammonium acetate buffer (pH 4.5) in the ratio of 30:20:50 (v/v) because it could resolve the peaks of Escitalopram with retention time at 2.8 min and Etizolam retention time at 5.7 min. The two peaks were symmetric and sufficiently resolved. System suitability was carried out by injecting 5 replicate injections of 100% concentration of Escitalopram and Etizolam. The resolution was found to be greater than 2 and the other parameters are presented in Table 1.

Specificity of the chromatographic method was tested by injecting mobile phase as blank and sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Escitalopram and Etizolam at 2.8min and 5.7min respectively without any interference. Thus the developed method was specific for analyzing the commercial formulations for Escitalopram and Etizolam. An optimized chromatogram with the retention times of Escitalopram and Etizolam was shown in the Figure 2.
The peak areas corresponding to the concentration range of Escitalopram 3.7 µg/mL and Etizolam 30-70 µg/ml prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for Escitalopram and Etizolam, respectively, with mean correlation coefficients (n=3) of 0.999 and higher, the representative calibration curve is shown in Figure 3. The regression analysis was given in Table 2.

Accuracy of the proposed method was assessed by standard addition method at 80%, 100% and 120% levels of recovery to the pre analyzed sample in triplicate. The recovery of the added standard to the sample was calculated and it was found to be 98.5-101.2 %w/w for Escitalopram and 98.4-100.8 %w/w for Etizolam respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in Table 3.

LOD and LOQ were calculated from the average slope and standard deviation of y intercepts of the calibration curve. Limit of detection for Escitalopram and Etizolam were 1.37 µg/mL and 0.66 µg/mL respectively whereas limit of quantitation of Escitalopram and Etizolam were 4.15 µg/mL and 2.00 µg/mL respectively indicating high sensitivity of the method. LOD and LOQ value was given in Table 2. The method is precise with a %RSD of less than 2 for both Escitalopram and Etizolam respectively. The results of intraday and inter day precision was given in Table 4. Robustness was carried out by change in the flow rate (±1mL/min), mobile phase variation (±5%) and variation in wavelength (±2 nm). Solution of 100% concentration of Escitalopram and Etizolam were analyzed in triplicate. The mean recovery values were 99.8 and 99.6 for Escitalopram and Etizolam respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in Table 6.

Table 6: Assay of Marketed samples for Escitalopram and Etizolam by proposed method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount Claimed in mg per Tablet</th>
<th>Estimated Amount in mg/tablet</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escitalopram</td>
<td>0.5</td>
<td>0.495</td>
<td>99.8</td>
</tr>
<tr>
<td>Etizolam</td>
<td>5</td>
<td>4.98</td>
<td>99.6</td>
</tr>
</tbody>
</table>

CONCLUSION
The proposed RP-HPLC method for simultaneous assay Escitalopram and Etizolam in combined dosage forms was validated, and found to be applicable for routine quantitative analysis of Escitalopram and Etizolam. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of Escitalopram and Etizolam with no interference from other formulation excipients. Therefore, this method can be employed for the routine analysis for simultaneous estimation Escitalopram and Etizolam in quality control of formulations and also in the dissolution studies.

REFERENCES
11. Fatema K, Rahman MZ, Biswas S and Akter S. Development of UV Spectroscopic Method For Nefopam And Escitalopram As INN Drugs In Tablet Dosage Form,


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Conflict of interest: None Declared