Development Of Validated Analytical Method for Simultaneous Estimation of Antianxiety Drugs by Liquid Chromatography with Qbd Approach

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ABSTRACT: The simple isocratic reverse-phase high-performance liquid chromatographic technique (RP-HPLC) used in this article was created for the simultaneous measurement of clonazepam (CZP) and escitalopram oxalate (EST) in pharmaceuticals and active pharmaceutical ingredients. As a stationary phase, a C18 column (id 4.6x150mm) was used to separate the two medicines. The mobile phase was a combination of 37:63 v/v isocratic methanol, and Formic Acid buffer (pH 4.2 adjusted by formic acid). PDA detectors operating at 255 nm were used for detection. Escitalopram Oxalate and Clonazepam were separated by a single chromatographic run with (RT = 3.979 minutes and 6.085 minutes, respectively). Escitalopram Oxalate had a satisfactory linear relationship, as evidenced by the calibration plot's r² = 0.998, r² = 0.999 for Clonazepam in the concentration range of 5 g/ml to 25 g/ml, and for Escitalopram Oxalate in the concentration range of 50 g/ml to 250 g/ml. Escitalopram Oxalate and Clonazepam both had retrievals of 99.45% and 99.49%, respectively. As per ICH guidelines for linearity, precision, accuracy, specificity, limit of detection, limit of quantification, robustness, and stability, the developed analytical technique was validated and confirmed to be acceptable. Escitalopram Oxalate and Clonazepam were subjected to a variety of stress conditions, including acid, base, heat, photolytic, and oxidative degradation, and the peaks of the degraded product could be reliably distinguished from the peaks of the pure medication. This technique is simple, efficient, and suitable for standard quality control analysis.

Keywords: Reverse Phase HPLC; Escitalopram Oxalate; Clonazepam; Validation; Degradation study.

INTRODUCTION:

Escitalopram Oxalate (EST): Chemically, escitalopram oxalate (EST) is known as S-(+)-1-[3-(dimethyl amino) propyl]. The S-enantiomer of racemic citalopram is 1-(p-fluro-phenyl)-5-phthalancarbonitrile oxalate, which belongs to the class of substances known as antidepressants.[1] Escitalopram is spontaneously soluble in DMSO, methanol, and to a lesser extent in water and ethanol. Escitalopram is hardly soluble in ethyl acetate but insoluble in heptane. Escitalopram oxalate belongs to the class of oral selective serotonin reuptake inhibitors (SSRI), and studies in vitro and in vivo have demonstrated its strong potency. Escitalopram is primarily used to treat severe depressive disorder and generalised anxiety disorder. Escitalopram functions by specifically competitively inhibiting the membrane serotonin transporter.[2] According to research, Escitalopram is the most highly selective medication in its class and is more than twice as strong as citalopram. Liquid chromatography combined with mass spectrometry is one of the analytical techniques that have been established for the determination of escitalopram oxalate in pharmaceutical formulations and/or biological fluids.[3]

Structure of Escitalopram Oxalate (Figure 1)

Clonazepam (CZP): The benzodiazepine derivative clonazepam (CZP) (Figure 1b), which is linked to diazepam, has different antiepileptic characteristics.[4] In BP and USP, it is legitimate. Clonazepam is a chemical compound with the chemical name 5-(2-chlorophenyl)-1, 3-dihydro- 7-nitro-2H-1,4-benzodiazepine-2-one. The powder form of clonazepam is a pale yellow, crystalline substance with hardly any odour. It is inaccessible to water but quite readily soluble in acetone, ethanol, and methanol.[5] The commercial medicine clonazepam is well known for treating depression brought on by anxiety and is also widely used to treat seizure and anxiety disorders. It functions by potentiating the effects of GABA through allosteric interactions between central
benzodiazepine receptors and gamma-aminobutyric acid (GABA) receptors. As an inhibitory neurotransmitter, GABA contributes to a greater degree of ascending reticular activating system inhibition (RAS).[6]

![Structure of Clonazepam (Figure2)](image)

The review of the literature reveals the availability of analytical techniques for the quantitative estimation of clonazepam (CZP) and escitalopram oxalate (EST) alone or in combination with other drugs, typically using chromatographic techniques with various detectors, such as electrochemical[7] or mass spectrometry detection fluorescence, gas chromatography with electron-capture, or mass spectrometry detection. In the current study, a method for simultaneously estimating EST and CZP in active pharmaceutical ingredients and commercial tablet formulations was developed and validated.[8] The created procedure was successfully used for the standard analysis of EST and CZP in bulk and sold tablet formulation.[9]

MATERIALS AND METHODS: As a gift sample from R.S.I.T.C. Jalgaon, Maharashtra, India, we obtained standards of escitalopram oxalate and clonazepam as well as commercial formulations. All of the chemicals, solvents, and reagents were purchased from R.S.I.T.C. Jalgaon Lab and were HPLC and analytical quality, milli-Q 0.45 Millipore nylon filter used for water purification. The commercially available formulation (REXIPRA PLUS INTAS) that was employed for the analysis was purchased from a retail pharmacy.

Instrumentation and chromatographic conditions: Agilent(1100) HPLC Gradient System DAD detector with, auto sampler, online degasser, sampler cooler, and DAD detector was used to conduct the analysis. Using Chemstation (10.04) software, operation data collecting and analysis were carried out. For separation, a C18 analytical column (4.6mm x 150mm ) was employed. At an isocratic ratio of 37:63 v/v, the isocratic elution was carried out using the mobile phase of methanol, and 0.1% Formic acid buffer (pH 4.2). At 255 nm, a PDA detector was used for detection 1.0 ml per minute of flow produced a back pressure of about 1039 psi. Formic acid was used to bring the pH of the buffer to 4.2, Formic Acid was dissolved in 400 ml of water to create the mobile phase buffer. Before to usage, mobile phase was vacuum-filtered using a 0.45-micron Millipore nylon filter and ultrasonically treated for 10 minutes. The column was kept at 35°C, detection was done at 255 nm using a PDA detector, and 20 µl injection volume was employed for analysis.

Standard solutions and calibration graphs forstandardization: 50 mg of EST and 5 mg of CZP working standards were individually weighed and placed into corresponding 100 ml volumetric flasks for stock standard solutions. Moreover, 10 ml of mobile phase was added to each volumetric flask, sonicated for 5 minutes, and used to create the volume. To prepare standard concentrations of 5000 µg/ml and 500 µg/ml, respectively, using mobile phase, aliquots of standard sub-stock solutions (0.1ml) of EST and CZP were properly pipetted out in 25 ml volumetric flasks.

Sample preparation:
The sample was made by precisely weighing 20 tablets of the EST and CZP combination in a mortar and pestle. Transfer the sample powder to a 50 ml volumetric flask after precisely weighing it to be approximately 1.015 gm of CZP/EST. The samples were sonicated in 5 ml of water for 10 minutes, followed by the addition of 30 ml of mobile phase, which was then shaken to dissolve and create volume. For estimating, 0.45 Millipore nylon filter paper was employed to filter the aforementioned sample solutions.

For Escitalopram:
Dilute 1 ml of the filtered solution with mobile phase and add it to a 10 ml volumetric flask for escitalopram.

Procedure: Measure area of both standard and sample and calculate the result by comparison. Each mg of Escitalopram oxalate eq. to 0.782751 mg of Escitalopram.

METHOD VALIDATION:
System Suitability Test: Using six replicate injections of a reference solution of EST and CZP, the system suitability test was carried out to determine the accuracy of the system for the analysis. The number of theoretical plates, retention time, peak area, and tailing factor were the criteria which have been measured.[10]

Specificity: The ability of a method to predict the analyte's reaction in the presence of its degradation products and probable contaminants is known as specificity. By utilising the proper dilutions and taking chromatograms, researchers were able to estimate how much medication was present.[11]

Linearity and range: The preparation of calibration curves involved graphing the concentrations of EST and CZP against their peaks. Using five calibration levels of 80%, 100% and 120% (i.e., 5,10,15, and 20 g/ml for EST and 0.5,1,1.5,2, and 12.5 g/ml for CZP), it was validated from 80% to 120% of standards concentration. Data were evaluated using the linear regression method.[12]
**Precision:** For the purposes of precision analysis of the analytical method, three duplicates of the standard solution were employed with various concentrations. The answers' repeatability (intraday precision) and intermediate precision (inter day precision) served as evidence.[13]

**Accuracy:** It gauges how closely experimental values match the real value. The EST/CZP (10mg/0.5mg) samples that had previously been examined were spiked with additional amounts of 80%, 100%, and 120% of the usual EST/CZP, and the suggested approach was employed to reassess the mixes. Several replicas of the experiment have been resorted successfully. For each concentration, RSD (%) and standard error mean (%) were computed. In the formulations, varied levels of drug recovery were guaranteed.[14]

**Limit of detection (LOD) and Limit of Quantitation (LOQ):** According to ICH guidelines, the smallest concentration of an analyte that can be accurately detected by an analytical process is referred to as the limit of detection (LOD) or limit of quantitation (LOQ). LOD was calculated through continual detection of the analyte at the minimum level using the visual evaluation method. The lowest amount of standard analytes that can be determined in a reproducible manner with respectable precision and accuracy were referred to as LOQs.[15]

**Robustness:** The ability of the analytical technique to remain unaffected by slight but intentional modifications to method parameters was referred to as robustness. By examining the effects of subtle and intentional adjustments made to chromatographic parameters such as column temperature, flow rate, pH, and mobile phase composition, robustness was examined.[16]

**Assay of marketed preparation:** The prepared sample preparations of EST and CZP were tested on HPLC and percentage assay was calculated using following formulas.[17]

\[
\text{Area of sample} \times \text{Concentration of standard} \times \text{Average weight of tablet} \times 100 = \% \text{ assay of API}
\]

**Forced Degradation Study:** A crucial step in the creation of a stability signalling approach is the forced degradation study. This investigation supported important information regarding the drug compounds' and drug products' potential storage-related pathways of degradation. Stress testing revealed that the technique was particular to EST and CZP.[18]

**Acid Hydrolysis:** Standards of EST (100 mg) and CZP (100 mg) were precisely weighed and put into three sets of 25 ml round bottom flasks for acid hydrolysis. All flasks received around 0.1 ml of 0.1N HCl, which was added and refluxed for 45 minutes at 80°C over a heated mantle.[19]

**Alkali Hydrolysis:** The standards for EST (100 mg) and CZP (100 mg) were precisely weighed and placed into three flasks. Each flask received 0.1N NaOH, which was added, and all were refluxed for 60 minutes at 80°C. [20]

**Oxidative Degradation:** Standards of EST (100mg) and CZP (100mg) were accurately weighed and transferred into three sets of 250ml round bottom flasks. About 5ml of 3% H2O2 was added to all flasks and refluxed on heated mantle for 2 hr. at 80°C.[21]

**RESULTS AND DISCUSSION:** Method optimisation: In the current investigation, the reversed-phase LC technique was used to separate the two analytes. The mobile phase employed in the study is made up of a combination of methanol and aqueous buffer, which are all organic solvents. Initially, the column employed as the stationary phase was a C-18 column (4.6x105mm) methanol and 0.1% Formic acid were combined in the mobile phase at an isocratic ratio of 37:63 v/v. At 255 nm, a PDA detector was used for detection, which demonstrated acceptable resolution between EST and CZP. The impact of altering the mobile phase's composition and pH (which can range from pH 3-6) on the peak asymmetry, theoretical plates, retention time, capacity factor (k'), and resolution was investigated in order to optimise the chromatographic parameters. The selection of the mobile phase's methanol: buffer: 37:63 v/v composition and Formic acid (pH 4.2) was made with the goal of achieving a strong baseline, acceptable separation, and sharp peaks in the shortest amount of run time. The PDA detector uses a 255 nm detection wavelength. The injection volume was 20µl, and the column temperature was maintained at 35°C for a 15-minute run.

**Optimized Chromatographic Condition:** The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

<table>
<thead>
<tr>
<th>HPLC</th>
<th>Agilent (1100) Gradient System DAD Detector with autosampler.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Software</td>
<td>Chemstation (10.04)</td>
</tr>
<tr>
<td>Column</td>
<td>150 mm length</td>
</tr>
<tr>
<td>Particle size packing</td>
<td>2.5 µm</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>RP C-18 (Agilent)</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Methanol : 0.1% Formic Acid (pH-4.2)</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>255 nm</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 D.C</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20µl</td>
</tr>
</tbody>
</table>
**ESC**

\[ y = 84.465x + 1002.4 \]

\[ R^2 = 0.9983 \]

**CLO**

\[ y = 85.547x + 3.27 \]

\[ R^2 = 0.9983 \]
### Linearity plot of Accuracy Recovery Studies

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (µg/ml)</th>
<th>Amount added (%)</th>
<th>Amount Recovered (µg/ml) ±SD</th>
<th>Recovery (%)</th>
<th>Average Recovery (%)</th>
<th>SEM</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EST</td>
<td>50</td>
<td>80</td>
<td>40</td>
<td>39.94</td>
<td>99.84</td>
<td>0.2</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>50</td>
<td>-16.83</td>
<td>-33.68</td>
<td>0.04</td>
<td>0.09</td>
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<tr>
<td></td>
<td></td>
<td>120</td>
<td>60</td>
<td>59.63</td>
<td>99.40</td>
<td>0.09</td>
<td>0.16</td>
</tr>
<tr>
<td>CZP</td>
<td>5</td>
<td>80</td>
<td>4</td>
<td>4.01</td>
<td>100.36</td>
<td>0.017</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>5</td>
<td>4.978</td>
<td>99.56</td>
<td>0.005</td>
<td>0.11</td>
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<tr>
<td></td>
<td></td>
<td>120</td>
<td>6</td>
<td>6.013</td>
<td>100.22</td>
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</tr>
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</table>

### Results of LOD and LOQ

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EST (µg/ml)</th>
<th>CLO (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.88</td>
<td>0.02</td>
</tr>
<tr>
<td>LOQ</td>
<td>2.66</td>
<td>0.08</td>
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</table>

### Acetic Acid Degradation of EST & CLO

![Acetic Acid Degradation Chart](chart)
CONCLUSION: For estimation and measurement of EST and CZP as tablet dosage forms in combination, in accordance with ICH recommendations, a validated stability indicating Reverse phase HPLC method has been devised. The method is inexpensive and easy to prepare. The verified outcomes demonstrated the specificity, sensitivity, linearity, precision, accuracy, and robustness of this methodology. It can be concluded that the pharmaceutical industry may effectively use this newly developed RP-HPLC method for regular analysis for the estimate of combination of EST and CZP in tablet as well as bulk dose form. The methodology for the intended application was supported by degradation studies. As an outcome, the proposed methodology can be used to examine various pharmaceuticals on a routine basis.

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REFERENCES:


