

# Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Escitalopram Oxalate and Clonazepam

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The objective of the current study was to develop a validated, specific stability-indicating reversed-phase liquid chromatographic (LC) method for the quantitative determination of escitalopram oxalate and clonazepam and their related substances in bulk drugs and pharmaceutical dosage forms in the presence of degradation products. Forced degradation studies were performed on the pure drugs of escitalopram oxalate and clonazepam, as per the stress conditions prescribed by the International Conference on Harmonization (ICH) using acid, base, oxidation, thermal stress and photolytic degradation to show the stability-indicating power of the method. Significant degradation was observed during acid and alkaline hydrolysis and no degradation was observed in other stress conditions. The chromatographic method was optimized using the samples generated from forced degradation studies. Good resolution between the peaks corresponded to the active pharmaceutical ingredients, escitalopram oxalate and clonazepam, and degradation products from the analyte were achieved on an ODS Hypersil C18 column (250 × 4.6 mm) using a mobile phase consisting of a mixture of acetonitrile–50 mM phosphate buffer + 10 mM triethylamine (70:30, v/v). The detection was conducted at 268 nm. The limit of detection and the limit of quantitation for escitalopram oxalate and clonazepam were established. The stress test solutions were assayed against the qualified working standards of escitalopram oxalate and clonazepam, which indicated that the developed LC method was stability-indicating. Validation of the developed LC method was conducted as per ICH requirements. The developed LC method was found to be suitable to check the quality of bulk samples of escitalopram oxalate and clonazepam.

## Introduction

Escitalopram oxalate (ESC) is chemically *S*-(+) 1-[3-(dimethylamino)propyl]-1-(*p*-fluorophenyl)-5-phthalan carbonitrile and clonazepam (CLO) is 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one (1) (Figure 1). ESC is a selective serotonin reuptake inhibitor (SSRI). It is the pure *S*-enantiomer (single isomer) of the racemic bicyclic phthalane derivative of citalopram (2). The antidepressant action of ESC, the *S*-enantiomer of racemic citalopram, is presumed to be linked to the potential of serotonergic activity in the central nervous system (CNS) neuronal reuptake of serotonin (5HT) (2, 3). ESC is at least 100-fold more potent than the *R*-enantiomer with respect to its inhibition of the 5HT reuptake and neuronal firing rate. CLO is a benzodiazepine derivative. It is a highly potent anticonvulsant, amnesic and anxiolytic. It has shown

itself to be useful as a short-term adjunct to SSRI treatments in clinical depression and obsessive compulsive disorder, and in combination is superior to SSRI treatments alone (3).

A literature survey reveals the simultaneous estimation of ESC and CLO by high-performance liquid chromatography (4) in plasma for pharmacokinetic studies using ultraviolet (UV) detection. Analyses have also been described of ESC and CLO in pharmaceutical preparations by high-performance thin-layer chromatography (HPTLC) (5, 6) and by spectrophotometrics (7, 8). The high-performance liquid chromatography mass spectrometric method (HPLC–ESI-MS) has been used to determine CLO in human plasma (9). Thus far, few articles have been published for the determination of ESC and CLO in metabolites and biological fluids (10). Most of these reported methods involve troublesome mobile phases (buffers) and difficult detection methods (fluorescence, HPTLC), and hence, are difficult to use for routine analysis. All of these methods were developed for ESC and CLO in biological fluids, and no stability-indicating methods have been published for the determination of ESC and CLO in bulk samples in the presence of degradation products and process-related impurities. The present research work was to develop a single, suitable stability-indicating LC method for the determination of ESC and CLO and their related substances, and the developed LC method was validated with respect to specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy and robustness. Forced degradation studies were performed on the drug substance to show the stability-indicating nature of the method and to ensure its compliance in accordance with International Conference on Harmonization (ICH) guidelines (11–14).

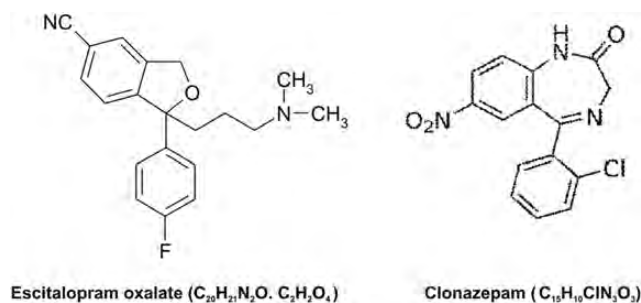


Figure 1. Structural formulae for ESC and CLO.

## Experimental

### Chemicals and reagents

Samples of ESC and CLO pure drugs were received from Bulk Actives, Unit-II of Dr. Reddy's Laboratories (Hyderabad, India). HPLC-grade methanol and acetonitrile were purchased from Rankem (Mumbai, India). Ortho-phosphoric acid was purchased from Qualigens Fine Chemicals (Mumbai, India). Potassium dihydrogen orthophosphate dihydrate was purchased from Qualigens Fine Chemicals. Triethylamine was purchased from Loba Chemie (Mumbai, India). HPLC-grade water was prepared by using a Millipore Milli-Q Plus purification system.

### Equipments

The LC method development, validation and forced degradation (stress) studies were conducted using a Shimadzu LC-10ATvp HPLC SPD-10AVp Variable UV-VIS detector with Shimadzu class CSW software.

### Chromatographic conditions

The chromatographic separations were achieved on an ODS Hypersil C18 column, 250 × 4.6 mm i.d. with 5 µm particle size, using a mobile phase composed of acetonitrile as solvent A and 50 mM phosphate buffer with 10.0 mM triethylamine as solvent B, pH 4.3, adjusted with O-phosphoric acid in the ratio of 70:30 (v/v) in isocratic mode. The column temperature was maintained at 40°C and the detection was conducted at 268 nm. The flow rate was kept at 1.5 mL/min and the injection volume was 20 µL.

### Preparation of standard solutions

For ESC, an accurately weighed quantity of 63.87 mg of ESC (i.e., equivalent to 50 mg of ESC) was dissolved in 25.0 mL of methanol. The volume was completed to 50.0 mL with methanol. A sample (2.5 mL) of the resultant solution was pipetted into a 10.0 mL volumetric flask. The volume was completed with mobile phase (concentration: 25.0 µg/mL).

For CLO, an accurately weighed quantity of approximately 50 mg of CLO was dissolved in methanol and diluted to 50.0 mL. Five milliliters of the resultant solution was diluted to 50.0 mL with the methanol. Further dilutions were conducted by adding 2.5 mL of the resultant solution to 10.0 mL with mobile phase (concentration: 2.5 µg/mL).

For the mixed standard stock solution, accurately weighed quantities of ESC (63.87 mg) and CLO (5 mg) were transferred to a 50.0 mL volumetric flask and dissolved in approximately 25.0 mL methanol, and the volume was completed with methanol.

For the working mixed standard solution, from the mixed standard stock solution, 2.5 mL was further diluted to 10.0 mL by the mobile phase to result in concentrations of 25.0 and 2.5 µg/mL for ESC and CLO, respectively.

### Preparation of sample solutions

Twenty tablets were accurately weighed and the average weight was calculated. The tablets were then crushed to obtain fine powder. An accurately weighed quantity of tablet powder equivalent to approximately 5.0 mg of ESC and 0.5 mg of CLO was transferred to a 50.0 mL volumetric flask, sonicated with

**Table I**  
Results of Degradation Study

Number	Degradation condition	Retention times of analyte and degradation products (min)			Percent degradation of drug (n = 5)	
		ESC	CLO	Degradation product	ESC	CLO
1	Untreated stock solution	6.66	9.44	—	—	—
2	Acid hydrolysis	6.64	9.44	5.90	0.0	84.38
3	Base hydrolysis	6.64	9.44	7.94	0.0	68.75
4	Oxidation	6.65	9.43	—	0.0	0.00
5	Thermal degradation	6.66	9.44	—	0.0	0.00
6	Photolytic degradation	6.66	9.44	—	0.0	0.00

25.0 mL methanol for 10 min and then shaken for 30 min. The volume was completed with methanol and filtered through Whatman Grade I filter paper.

The chromatographic conditions were set as per the optimized parameters and the mobile phase was allowed to equilibrate with the stationary phase. After equilibration of the stationary phase, 20 µL of five replicates of the standard solution and sample solution were injected and chromatograms were recorded.

### Method Validation

#### Stress studies and specificity

Stress testing of drug substances can help to identify the likely degradation products, which can, in turn, help to establish the degradation pathways and the intrinsic stability of the molecules. Specificity is the ability of the method to measure the responses of the analyte in the presence of its related substances (15). All stress degradation studies were performed at initial drug concentrations of 25.0 and 2.5 µg/mL for ESC and CLO, respectively. Acid hydrolysis was performed in 0.1N HCl at 50°C for 24 h. The study in basic solution was conducted in 0.1N NaOH at 50°C for 24 h. Oxidation studies were conducted at ambient temperature in 3% hydrogen peroxide for 24 h. For photodegradation studies, the drug sample was exposed to light for 24 h in a UV chamber. The drug sample was exposed to dry heat at 60°C for 24 h. Samples were withdrawn at appropriate times and subjected to LC analysis after suitable dilution to evaluate the ability of the proposed method to separate ESC and CLO from their degradation products. A photodiodearray (PDA) detector was employed to ensure the homogeneity and purity of ESC and CLO peaks in all stressed sample solutions. Assessment of the mass balance in the degraded samples was conducted to confirm that the amount of degraded product detected in stressed samples matched with the amount present before the stress was applied. Quantitative determination of ESC and CLO was conducted in all stressed samples against qualified working standards, which is tabulated in Table I.

#### LOD and LOQ

The LOD is the lowest analyte concentration that can be detected. LOQ is the lowest analyte concentration that can be quantified with acceptable accuracy and precision. LOD and LOQ were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively (15, 16), by injecting a series of diluted solutions with known concentrations.

### **Linearity**

Linearity test solutions of ESC for the assay method were prepared at concentration levels of 5.0 to 25.0 µg/mL; concentration levels for CLO were 0.5 to 2.5 µg/mL. The peak area versus concentration data were calculated by least-squares linear regression analysis. Linearity test solutions for ESC and CLO were prepared by diluting the stock solution to the required concentrations. The solutions were prepared at five concentration levels from LOQ. The calibration curve was drawn by plotting the peak areas of ESC and CLO versus their corresponding concentrations. A linearity test was performed for two consecutive days in the same concentration range for both the assay and the related substance method. The correlation coefficient of the calibration curve was calculated.

### **Precision**

The assay method precision was evaluated by conducting five independent assays of test samples of ESC and CLO against qualified working standards and calculating the percentage of relative standard deviation (RSD). The precision of the related substance method was checked by injecting five individual preparations of ESC and CLO spiked at three levels. The RSD percentage of the area was calculated for each substance. The intermediate precision of the method was also verified using different analysts and different days.

### **Accuracy**

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the value found (17). The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e., 80, 100 and 120% of the label claim. The percentage recoveries were calculated from the slope and Y-intercept of the calibration curve. Standard addition and recovery experiments were conducted to determine the accuracy of ESC and CLO for the quantification of drug in the samples. The study was conducted in triplicate by spiking each drug. The percentage recoveries for drugs were calculated from the slope and Y-intercept of the calibration curve.

### **Selectivity**

The selectivity of the method was established from the resolution of the drug peak from the nearest peak and among all the other peaks. All degradants were separated from each other and from the analyte with acceptable resolution.

### **Robustness**

To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between ESC and CLO was evaluated. To study the effect of flow rate on the resolution, the flow rate was altered by 0.2 units, i.e., 1.3 and 1.7 mL/min from the actual flow, 1.5 mL/min. The effect of column temperature on resolution was studied at 35 and 45°C instead of 40°C. The effect of pH was studied by changing pH by 0.5 units from the value of 4.3, and the remaining method conditions were constant.

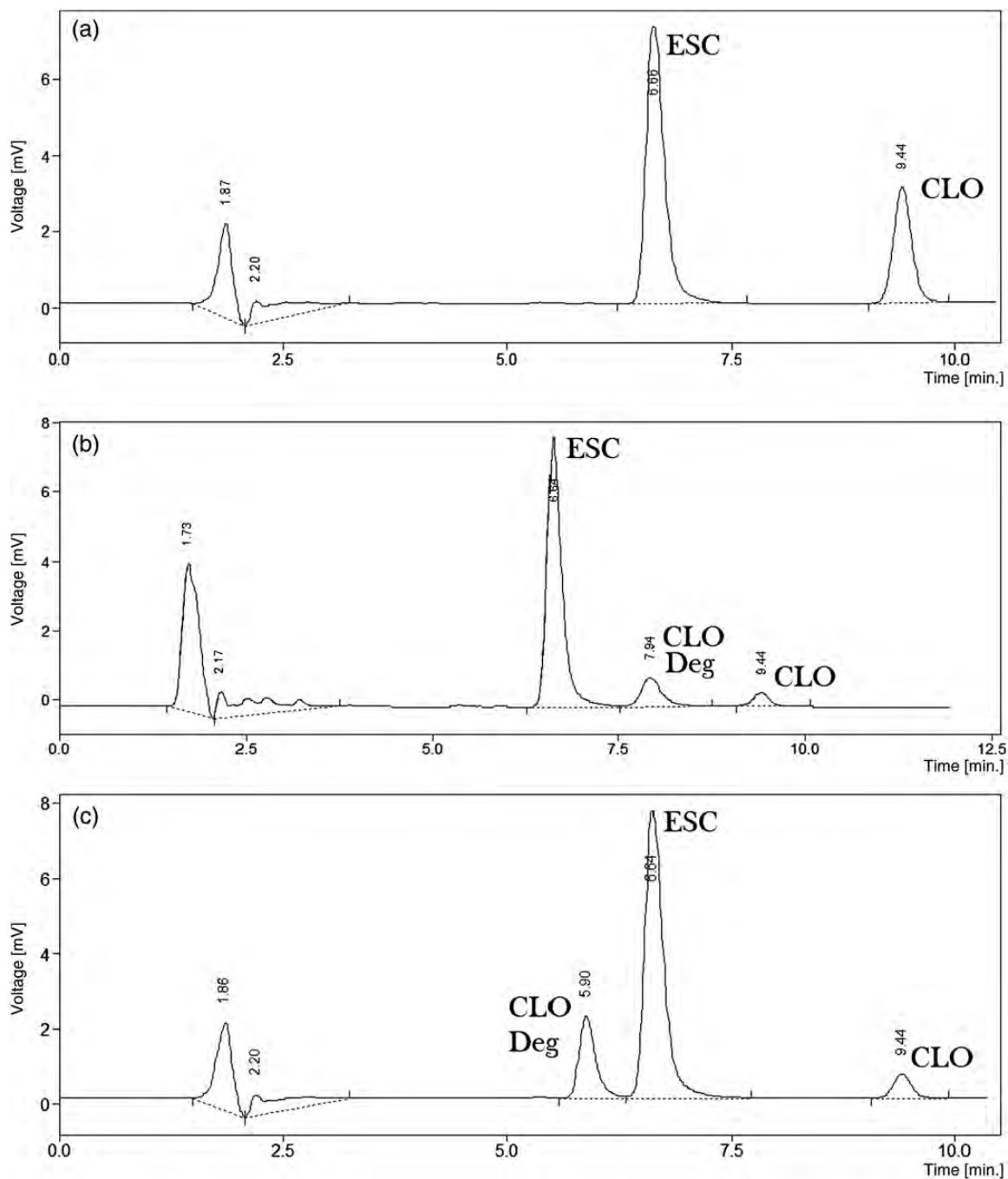
## **Results and Discussion**

### **Optimization of chromatographic conditions**

The primary target of the chromatographic method is to separate ESC, CLO and the degradation products generated from the analyte peaks during stress studies. Degradation products were co-eluted by using different stationary phases like C8, cyno and phenyl, and different mobile phases containing buffers like phosphate, sulphate and acetate with different pHs (3–8), and by using organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. Apart from the co-elution of the analyte, poor peak shapes were also noticed for some degradation products. Sodium dihydrogen orthophosphate buffer, pH 6.0, and methanol at a flow of 1.0 mL/min were chosen for initial trial with a C18 stationary phase column of 250 mm length × 4.6 mm i.d. and 5 µm particle size. When the sample was injected, the resolution between the degradation product and the analyte was poor. To achieve good resolution of the degradants from the analyte, triethyl amine was added to the phosphate buffer from 5 to 10 mM and the pH was adjusted to 5.0 with ortho phosphoric acid at each level of triethyl amine. Sample solution was injected again, and at a level of 10 mM triethyl amine, the resolution was good among degradants and analytes. At low concentrations of triethyl amine, the resolution between acidic degradants was poor. At a level of triethyl amine of 10 mM at pH 6.0, all degradation products were well separated from each other and from the analyte. The effect of buffer pH was also studied under the preceding conditions and it was found that at higher and lower pH, the tailing of the ESC and CLO peaks was more pronounced and resolution was poor between the analytes and degradants. The effect of buffer concentration on the retention of ESC and CLO and their degradants was also studied. At a low concentration of buffer, the retention times of the analytes and degradants were very high. To decrease the retention times of the analytes and impurities, the buffer concentration was increased to 50 mM without changing any other conditions. At these chromatographic conditions, all degradants were satisfactorily separated from each other, ESC and CLO. The effect of pH was also studied; various pH compositions were tried, ranging from 3.0 to 5.0 with ortho phosphoric acid. At pH 3.0, tailing in the peak of ESC was observed when the buffer and triethyl amine concentrations were fixed. The pH of the solution was optimized at the pH value  $4.3 \pm 0.05$ , which resulted in good resolution of ESC and CLO and their degraded products. The composition of the organic modifier was optimized with acetonitrile at 70% and buffer at 30%. Accelerated and long-term stability study results as per ICH Q1A (R2) for ESC and CLO were generated by using the developed LC method and the results were well within the limits, which further confirms that the developed LC method is stability-indicating.

### **Results of forced degradation**

Accurately weighed quantities of powdered tablets, equivalent to approximately 5.0 mg of ESC and CLO, were transferred to five different 50.0 mL volumetric flasks. The samples were then exposed to stress conditions that



**Figure 2.** Typical HPLC chromatograms: untreated sample solution (A); solution after exposure to 0.1N NaOH (B); solution after exposure to 0.1N HCl (C).

included 1.0 mL of 0.1N HCl for 50°C, 1.0 mL of 0.1N NaOH for 50°C, 1.0 mL of 3% H<sub>2</sub>O<sub>2</sub> for 50°C, a UV chamber at 265 nm and dry heat for 60°C and 24 h. ESC and CLO were stable under photolytic, oxidation and thermal conditions. No extra peaks were obtained under the described conditions and a chromatogram was obtained, as shown in Figure 2(a). Significant degradation of the drug substances was observed under acidic and alkaline stress, leading to the formation of some unknown degradation product at retention times 5.90 and 7.94, respectively. The peaks at 5.90

and 7.94 were obtained from the acidic and alkaline degradation of CLO, respectively. Peak purity was determined from PDA detection, which showed that the peak at 5.90 was obtained from the degradation of CLO in acidic solution and the peak at 7.94 was obtained from the degradation of CLO in alkaline solution. The results of percent degradation of the samples are given in Table I. The assay of ESC and CLO was unaffected by the presence of degradation products, which confirms that the developed LC method is stability-indicating.

## Method Validation

### LOD and LOQ

The LODs of ESC and CLO were 1.030 and 0.101 µg/mL, respectively. The LOQs of ESC and CLO were 3.121 and 0.306 µg/mL, respectively (Table II).

### Linearity

Linear calibration plot for the assay method was obtained over the calibration ranges tested and the correlation coefficient obtained was greater than 0.998. Linearity was checked for the assay method over the same concentration range for two consecutive days. The results show that an excellent correlation existed between the peak area and concentration of the analyte (Table II).

### Precision

The percentage RSD of assay of ESC and CLO during a precision study of the assay method was well within  $\pm 1.0\%$ .

**Table II**  
Results of Linearity and Range

Number	Parameter	ESC	CLO
1	Linear dynamic range (µg/mL)	5-25	0.5-2.5
2	Slope	4.152	19.740
3	Y-intercept	1.680	-0.130
4	Correlation coefficient (r)	0.999	0.999
5	LOD (µg)	1.030	0.101
6	LOQ (µg)	3.121	0.306

**Table III**  
Results of Precision Study

Nexito Plus (average weight: 81.53 mg)

Number	Weight of tablet powder (mg)	Amount estimated (mg)		Percent of labeled claim	
		ESC	CLO	ESC	CLO
1	80.53	6.37	0.496	99.94	98.60
2	81.03	6.39	0.504	100.15	100.08
3	81.04	6.44	0.504	100.98	100.06
4	80.08	6.46	0.499	101.34	99.15
5	82.26	6.45	0.507	101.12	100.63
Mean				100.70	99.70
± SD				0.621	0.814
RSD (%)				0.617	0.816

**Table IV**  
Results of Recovery Study

Number	Recovery level (%)	Weight of tablet powder taken (mg)	Weight of pure drug added (mg)		Amount of drug recovered (mg)		Recovery (%)	
			ESC	CLO	ESC	CLO	ESC	CLO
			1	80	40.38	1.90	0.157	4.66
2	100	40.08	3.18	0.262	5.66	0.495	100.4	100.40
3	120	41.72	4.46	0.367	6.80	0.609	100.57	101.42
Mean							100.1	100.82
± SD							± 0.672	± 0.530
RSD (%)							0.671	0.525

The percentage RSDs for ESC and CLO were found to be 0.617 and 0.816 (Table III).

### Accuracy

The percentage recoveries ranged from 99.33 to 100.57 for ESC and 100.66 to 101.42 for CLO in bulk drug samples (Table IV).

### Application of the Developed LC Method to Stability Samples and Quality Monitoring of Escitalopram Oxalate and Clonazepam

Accelerated and long-term stability studies are conducted to establish the retest period or shelf-life of drug products, to determine the effects of storage conditions at different atmospheric conditions and to show that the method is stability-indicating (17). The results clearly indicate that the drugs were stable under long-term and accelerated conditions and there were no interferences of the related substance for ESC and CLO, which demonstrates that the developed LC method was stability-indicating and well-applied for drug stability studies and quality control monitoring of ESC and CLO.

### Conclusions

In this paper, a sensitive, specific, accurate, validated and well-defined stability-indicating LC method was described for the determination of ESC and CLO in the presence of degradation products. The behavior of ESC and CLO under various stress conditions was studied. All degradation products were satisfactorily separated from the drug substances, which demonstrates that the method is stability-indicating. The information presented here can be very useful for quality monitoring of bulk samples and formulations and to check the quality of drugs during stability studies.

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