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Development and validation of an RP-HPLC method for the simultaneous determination of Escitalopram Oxalate and Clonazepam in bulk and its pharmaceutical formulations

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ABSTRACT

A Simple, efficient and reproducible reverse phase high performance liquid chromatographic method was developed and validated for the Simultaneous determination of Escitalopram oxalate and Clonazepam in combined dosage form. The separation was effected on a Hypersil ODS C_{18} column (250mm X 4.6mm; 5µ) using a mobile phase mixture of buffer and acetonitrile in a ratio of 50:50 v/v at a flow rate of 1.0ml/min. The detection was made at 240nm. The retention time of Escitalopram oxalate and Clonazepam was found to be 2.840± 0.007min and 4.007±0.006 min. Calibration curve was linear over the concentration range of 20-120µg/ml and 1-6µg/ml for Escitalopram oxalate and Clonazepam. All the analytical validation parameters were determined and found in the limit as per ICH guidelines, which indicates the validity of the method. The developed method is also found to be precise, accurate, specific, robust and rapid for the simultaneous determination of Escitalopram oxalate and Clonazepam in tablet dosage forms.

Key Words: Buffer, Acetonitrile, Escitalopram oxalate, Clonazepam, Tablets, Hypersil C_{18} column, RP-HPLC.

INTRODUCTION

Escitalopram oxalate (Martindale,34th edition, 2005, 292.1) [(s)-1-[3-(dimethlyamino) propyl]-1-(4-flurophenyl)-1,3- dihydro isobenzofuran-5-carbonitrile oxalate] is a pure s- enantiomer of the racemic, bicyclic pthalates derivatives citalopram. Escitalopram is freely soluble in methanol and dimethlysulfoxide (DMSO), sparingly soluble in water and in ethanol, slightly soluble in ethyl acetate, insoluble in heptane. It is mainly used as an antidepressant agent. Clonazepam (Merck Index, 13th edition, 2002, 2413) [5-(o-chlorphenyl)-7-nitro-1H-1,4-benzodiazepin-2(3H)-one] is mainly used as anticonvulsant, muscle relaxant and anxiolytic agent. Clonazepam is slightly soluble in acetone, chloroform, acetic anhydride, hardly soluble in methanol, isopropanol, ether, almost insoluble in water. A Literature survey reveals that only a few methods based on RP-HPLC, Spectrophotometric, colorimetric methods were developed and validated for the simultaneous determination of Escitalopram oxalate and Clonazepam in combined dosage forms. Spectrophotometric determination of Escitalopram oxalate and Clonazepam using multi-component mode of analysis (Sharma *et al.*, 2010) is available. An isocratic chiral sensitive HPLC method was developed for the separation of Escitalopram oxalate drug substance (Nagarjuna *et al.*, 2006). HPLC assay of clonazepam in human plasma using a non-porous silica column is found (Nakamura *et al.*, 2004). Simultaneous HPTLC determination of Escitalopram oxalate and clonazepam in combined tablets (Dhavale *et al.*, 2008), spectrophotometric method for simultaneous Estimation of Escitalopram oxalate and clonazepam in tablet dosage forms (Kakde *et al.*, 2009), HPLC determination of

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clonazepam in plasma using solid-phase extraction (Sallustio *et al*., 1994), spectrophotometric and reverse phase high-performance liquid chromatographic methods for the determination of Escitalopram oxalate and clonazepam in combined tablet dosage forms (Gandhi *et al*., 2008), liquid chromatography-electrospray ionisation mass spectrometric method for the determination of Escitalopram in human plasma and its applications in bioequivalence study (Singh *et al*., 2004), analysis of clonazepam in tablet dosage forms using small bore HPLC (Spell and Stewart, 1998), colorimetric method for the estimation of esitalopram oxalate in tablet dosage forms (Vetrichelvan *et al*., 2010), Automated extraction and high- performance liquid chromatographic determination of serum clonazepam (Taylor *et al*., 1984) are few available related works. The present investigation describes a rapid, accurate and precise RP-HPLC method for the determination of Escitalopram oxalate and Clonazepam from bulk sample and pharmaceutical combined dosage forms since this drug is being marketed in domestic and international market. The method was validated as per ICH guidelines.

EXPERIMENTAL

Chromatographic Conditions

Shimadzu made high pressure liquid chromatographic instrument provided with LC 20 AD Pump and Prominence SPD 20A UV-deuterium detector and a Hypersil ODS C_{18} column (250 mm x 4.6 mm; 5µ) was employed in the study. A 20µL Hamilton injection syringe was used for sample injection. Data acquisition was performed by using Spinchrome software, Shimadzu Class VP version 6.12 SPS data system (Bhimanadhuni *et al*, 2012). HPLC grade acetonitrile, Methonal, water were purchased from E. Merck Co; Mumbai, India, and Potassium dihydrogen phosphate,ortho phosphoric acid AR grade were purchased from SD Fine Chem Mumbai, India were used in the study.

Drug Samples

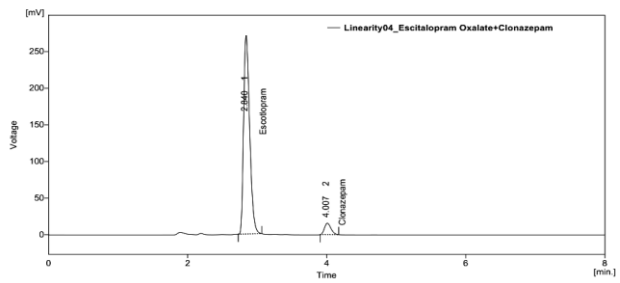
The reference samples of Escitalopram oxalate and Clonazepam was supplied by M/s Bio Leo Analytical Labs India Pvt. Ltd, Hyderabad, Andhra Pradesh, India, and branded formulation purchased from local market (Snudep plus®).

Mobile Phase

Accurately 13.6g of potassium dihydrogen phosphate was weighed and dissolved in 100ml of water and the volume was made up to 1000ml with water. pH was adjusted to 3.0±0.05 using dilute ortho phosphoric acid. The solution was filtered through 0.45µ membrane filter and was degassed. A freshly prepared binary mixture of buffer: Acetonitrile in a ratio of (50:50) V/V was used as the mobile phase. Methanol was used as diluent for preparing the working solution of the drug. The mobile phase was filtered through 0.05µ membrane filter and sonicated by using Power Sonicator, model no: 405, Hwashin Technology, Korea before use (Bhimanadhuni *et al*; 2012). The flow rate of the mobile phase was maintained at 1.0ml/min. The column temperature was maintained at 25°C and the detection of the drug was carried out at 240nm.

Stock and Working Standard Solution

50mg of Escitalopram oxalate was weighed accurately and transferred in to 50ml volumetric flask. The solution was sonicated and filtered through Whatman filter paper, resulting solution was diluted with the mobile phase to get a working standard solution. 2.5mg of Clonazepam was weighed accurately and transferred into 50ml volumetric flask and the solution was sonicated and filtered through Whatman filter paper, resulting solution was diluted with the mobile phase to get a working standard solution. Stock solutions of Escitalopram oxalate and Clonazepam (1mg/1ml) were prepared separately using mobile phase as solvent from this standard stock solutions, mixed standard solutions of different concentrations ranging from 20-120 µg/ml of Escitalopram oxalate and 1-6µg/ml of Clonazepam were prepared by taking suitable aliquots of working standard solution in different 10ml volumetric flasks and diluting up to the mark with the mobile phase. With the optimized chromatographic conditions, a steady base line was recorded. 20 micro liters of each mixed standard solution was injected 6 times and chromatograms were recorded. The retention time of Escitalopram oxalate and Clonazepam were found to be 2.840±0.007min and 4.007±0.006min respectively. Calibration curves were constructed by plotting the average peak areas against the respective concentrations and found be linear in the above range with the correlation coefficients (r^2) 0.99920



Result Table (Uncal - Linearity04_Escitalopram Oxalate+Clonazepam)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.840	1742.885	270.771	94.232
2	4.007	110.566	15.574	5.768
	Total	1853.451	286.345	100.000

Column Performance Table (From 50% - Linearity04_Escitalopram Oxalate+Clonazepam)

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	2.840	0.100	1.650	4468	-
2	4.007	0.103	1.435	8329	6.752

Figure 1: Chromatogram of Escitalopram oxalate 80µg/ml and Clonazepam 4µg/ml.

and 0.99918 for Escitalopram oxalate and clonazepam respectively. This regression equation was later used to estimate the amount of Escitalopram oxalate and clonazepam in pharmaceutical combined dosage forms. A representative chromatogram for the separation of Escitalopram oxalate and clonazepam is given in figure 1.

Analysis of Tablet

Twenty tablets (Containing both Escitalopram Oxalate and Clonazepam) were weighed and average weight was determined and finally powdered. Tablet powder equivalent to 20mg of Escitalopram oxalate and clonazepam was accurately weighed and transfer to 50ml volumetric flask. The contents were sonicated for about 15min for complete solubility of the drug after adding 25ml of mobile phase and the volume was made up to the mark with mobile phase. Then the mixture was filtered through a 0.45µ membrane filter. From the above solution 2.5ml aliquot was taken into a separate 10ml volumetric flask and diluted up to the volume with the mobile phase and mixed well. The above solution (20µL) was then injected Six times into the column and the peak areas were measured and the quantitation was carried out by keeping these values to the regression equation of corresponding calibration curve.

RESULTS AND DISCUSSION

The goal of this present study was aimed at developing a sensitive, precise and accurate HPLC method for the analysis of Escitalopram oxalate and clonazepam in its bulk and pharmaceutical combined dosage forms. In order to achieve optimum separation of the component peaks, mixtures of buffer with acetonitrile in different combinations were tested as mobile phase on a Hypersil C_{18} stationary phase. A binary mixture of buffer: acetonitrile in a proportion of 50:50 v/v was selected as the chromatographic peaks were well defined and resolved with no tailing. The retention time obtained for Escitalopram oxalate and clonazepam was found to be 2.840±0.007min and 4.007±0.006min. Each of the samples was injected Six times and the Sample retention times were observed in all cases. The peak areas of Escitalopram oxalate and clonazepam were reproducible as indicated by low coefficient of variation. Calibration curves were constructed by plotting the average peak areas against the respective concentrations and found be linear in the above range with the correla-

Table 1: Calibration data of the proposed method.

Sl. No.	Escitalopram oxalate					Clonazepam				
	Concentration (µg/ml)	peak area	Correlation coefficient (r^2)	Slope (m)	Intercept (b)	Concentration (µg/ml)	peak area	Correlation coefficient (r^2)	Slope (m)	Intercept (b)
1	20	560.978	0.99920	20.59	124.04	1	34.850	0.999188	26.03	7.025
2	40	911.535				2	57.401			
3	60	1370.185				3	83.692			
4	80	1742.885				4	110.566			
5	100	2224.455				5	140.301			
6	120	2581.977				6	161.984			

Table 2a: Accuracy data of Escitalopram oxalate (Triplicate values at 80, 100, 120 percent levels).

*Amount taken (µg)	*Amount found (µg)	*Percent recovery	*Mean percentage recovery
90	89.19	99.10	99.10
110	109.68	99.71	99.71
130	129.69	99.76	99.76

*Each value is a mean of three readings

Table 2b: Accuracy data of Clonazepam (Triplicate values at 80, 100, 120 percent levels).

*Amount taken (µg)	*Amount found (µg)	*Percent recovery	*Mean percentage recovery
4.5	4.49	99.95	99.95
5.5	5.46	99.41	99.41
6.5	6.47	99.59	99.59

*Each value is a mean of three readings

tion coefficients (r^2) 0.99920 and 0.99918 for Escitalopram oxalate and clonazepam respectively. The regression curve was constructed by linear regression fitting and its mathematical expression was Y =20.595X + 124.04 and 126.03X + 7.025 (Where Y gives peak area and X is the concentration of the drugs of Escitalopram oxalate and clonazepam). The regression characteristics are given in table 1. When Escitalopram oxalate and clonazepam solutions were analysed by the proposed method for finding out intra and inter-day variation, low co-efficient of variation was observed. The absence of additional peaks indicated non-interference of common excipients used in the tablets.

High recovery values obtained from the combined dosage form by the proposed method indicates the method is accurate. The drug content in tablets was quantified using the proposed analytical method are given in table 2a and 2b.

The deliberate changes in the method have not much affected the peak tailing, Theoretical plates and the percent assay. This indicated the robustness of the method. The robustness study results are presented in table 3. The lowest value of LOD and LOQ were obtained by the proposed method of Escitalopram oxalate and clonazepam indicates the sensitivity of the method. The standard solution of the drug was stable up to 24 hrs as the difference in percent assay during the above period is within limit system suitability parameters were studied with six replicates standard solution of the drug and the calculated parameters are within the acceptance criteria. The tailing factor and the number theoretical plate are in the acceptable limits. The system suitability results are shown in table 4.

The system precision was established by six replicate injections of the standard solution containing analytes of interest. The value of relative standard deviation of Escitalopram oxalate and clonazepam was found to be 0.261 and 0.485 within the limit, indicating the injection repeatability of the method. The method precision was established by carrying out the analysis six times using the proposed method. The relative standard deviation of Escitalopram oxalate and clonazepam was found to be 0.112 and 0.853 within the limit, indicating the injection repeatability of the method.

Table 3: Robustness Study.

Drug name	Variations	Chromatographic parameters				
		Retention time	Area	Height	Theoretical plates	Asymmetry
Escitalopram oxalate	Change in wave length at ± 2nm					
	wave length at 238nm	2.837	2507.148	405.076	4458	1.75
	wavelength at 242nm	2.837	2314.536	373.893	4458	1.70
	Change in flow rate at ±0.1ml/min					
	flow rate at 1.1ml/min	2.610	2203.232	380.307	4332	1.68
	Change in pH	2.957	3135.473	488.695	4536	1.80
Clonazepam	Change in wave length at ± 2nm					
	wave length at 238nm	3.997	160.023	24.550	7778	1.42
	wavelength at 242nm	3.997	151.185	22.879	7778	1.36
	Change in flow rate at ±0.1ml/min					
	flow rate at 1.1ml/min	3.667	140.356	22.693	7448	1.455
	Change in pH	4.163	190.244	28.640	8440	1.360

Table 4: System Suitability Parameters.

Parameters	Escitalopram oxalate	Clonazepam
Theoretical Plates (h)	4782	8329
Tailing factor (T)	1.650	1.360
LOD (µg/ml)	2.398	0.064
LOQ (µg/ml)	7.27	0.194
Resolution	-	6.865

The specificity of the HPLC method was determined by the complete separation of Escitalopram oxalate and clonazepam. When it was subjected to forced degradation as per ICH guidelines which was carried out with 0.1N HCL, Photolytic and Heat degradation at 105°C. The method does not permit detection of degradation product for Escitalopram oxalate and clonazepam. The results of specificity data for degradation study are given in table 5.

Hence it can be concluded that the proposed HPLC method is sensitive and reproducible for the analysis of Escitalopram oxalate and clonazepam in pharmaceutical combined dosage form with short analysis time of 8 min.

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Table 5: Forced Degradation.

Drug Name	Condition	Time in hours	Retention time (min)	Area	% Degradation	% of Active drug Present after Degradation
Escitalopram oxalate	Acid Degradation	24	2.847	2894.76	7.24	92.76
	Photolytic Degradation	24	2.843	1892.9	39.34	60.66
	Thermal Degradation	24	2.843	2275.33	27.09	72.91
Clonazepam	Acid Degradation	24	4.007	158.28	10.48	89.52
	Photolytic Degradation	24	4.007	121.549	31.25	68.75
	Thermal Degradation	24	4.01	146.12	17.36	82.64

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