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DEVELOPMENT AND VALIDATION OF UV SPECTROSCOPIC SIMULTENOUS METHOD FOR ZONISAMIDE AND CILOSTAZOL IN SYNTHETIC MIXTURE

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ABSTRACT

A simple, specific, accurate and precise Zero order derivative spectrophotometric method was developed and validated for simultaneous estimation of Zonisamide and Cilostazol in Synthetic Mixture. The wavelength of estimation for Zonisamide was 283.80 nm and for Cilostazol was 257.60 nm. Beer's law is obeyed in the concentration range of 4-20µg/ml and 4-20µg/ml and correlation coefficient of 0.9999 and 0.9999 for Zonisamide and Cilostazol respectively. The % recovery for Zonisamide and Cilostazol were found to be 100.62 % - 101.32 % and 101.48 - 101.76 % respectively. Intraday precision of Zonisamide and Cilostazol were found to be 0.113 – 0.308 and 0.077 – 0.178 % RSD and Interday precision were found to be 0.178 – 0.459 and 0.103 – 0.307 % RSD respectively. The proposed method was also evaluated by the Assay of Synthetic mixture containing Zonisamide and Cilostazol. The % Assay was found to be 100.69 ± 0.177 for Zonisamide and 100.05 ± 0.272 for Cilostazol. Validation of proposed methods was carried out according to ICH Q_2R_1Guidelines. The proposed methods were found accurate and reproducible for routine analysis of both the drugs in synthetic mixture.

KEYWORDS: Zonisamide, Cilostazol, Simultaneous equation method, UV Spectroscopy.

INTRODUCTION

Combination composition of Zonisamide and Cilostazol is used for treating Alzheimer and related disorder. Zonisamide binds to sodium channels and voltage sensitive calcium channels, which suppresses neuronal depolarization and hypersynchronization and muscular pain disorder observed in Zonisamide treatment of Alzheimer. Cilostazol is used to improve the symptoms of a certain blood flow problem in the legs (intermittent claudication). Claudication pain is caused by too little oxygen getting to the muscles[1-2]. Cilostazol can increase blood flow and the amount of oxygen that gets to the muscles Zonisamide and Cilostazol combination was Application on 23 Feb 2011. Patented by Pharnext, EP patent: EP2285374A1 combination with both drugs help to treatment of Alzheimer's disease and related disorder. Hence, there is a scope to develop analytical methods for Zonisamide and Cilostazol in combination[3].

Literature review reveals that, various analytical methods have been reported for the estimation of Zonisamide and Cilostazol in biological fluids, pharmaceutical formulation and bulk drug include UV spectrophotometric, High-performance liquid chromatography method (HPLC), Stability indicating RP-HPLC method, HPTLC method, TLC method, LC/MS/MS method and UPLC method in individual and/or in combination of other drug.

Literature review shows that, there is no reported method available for simultaneous estimation of both the drugs in combination. Therefore it is thought of interest to developed simple, accurate, precise and rapid methods for simultaneous estimation of Zonisamide and Cilostazol in combination[4-15].

MATERIAL AND METHOD

INSTRUMENT AND APPARATUS

Table: 1.1 Lists of Instrument and Apparatus

Component	Model/Software	Manufacturer
Double Beam UV-Visible Spectrophotometer	Shimadzu-1800, UV Probe 2.34	Shimadzu
Analytical Balance	Wensar	Wensar
Volumetric Flask	-	Borosil
Pipettes	-	Borosil
Beaker	-	Borosil

REAGENTS AND MATERIAL

All the Reagents and Solvents used were of AR or HPLC grades.

Table: 1.2 Working Standard API

Standard	Purpose	Source
Zonisamide	Analysis	ZCL Pvt. Ltd, G.I.D.C, Ankleshwar
Cilostazol	Analysis	Pure Chem Pvt. Ltd, G.I.D.C, Ankleshwar

PREPARATION OF SOLUTIONS

1. **Standard Stock Solution of Zonisamide**

   An accurately weighed quantity of Zonisamide (10 mg) was transferred into 100 ml volumetric flask and dissolved with 25 ml with methanol, Sonicated for 15 min. diluted up to the mark with Methanol to obtain standard solution having concentration of ZON (100µg/ml).

2. **Standard Stock Solution of Cilostazol**

   An accurately weighed quantity of Cilostazol (10 mg) was transferred into 100 ml volumetric flask and dissolved with 25 ml with methanol, Sonicated for 15 min. diluted up to the mark with Methanol to obtain standard solution having concentration of CIL (100µg/ml).

3. **Working standard solution of Zonisamide**

   From 100µg/ml solution of Zonisamide 0.4, 0.8, 1.2, 1.6 and 2.0 ml was transferred into 10 ml volumetric flask and adjust with methanol up to mark to get the final concentration of 4, 8, 12, 16 and 20µg/ml.

4. **Working standard solution of Cilostazol**

   From 100µg/ml solution of Cilostazol 0.4, 0.8, 1.2, 1.6 and 2.0 ml was transferred into 10 ml volumetric flask and adjust with methanol up to mark to get the final concentration of 4, 8, 12, 16 and 20µg/ml.

SIMULTANEOUS EQUATION METHOD[16]

To determine wavelength for measurement, standard spectra Zonisamide and Cilostazol was scanned between **200-400 nm against Methanol**. The method was based on the measurement of absorbance of Zonisamide and Cilostazol at **283.80nm** and **257.60nm** respectively. This method obeyed Beer's law in the concentration range of **4-20µg/ml** for Zonisamide and **4-20µg/ml** for Cilostazol.

$$Cx = A_2ay_1 - A_1ay_2 / ay_1ax_2 - ay_2ax_1$$

$$Cy = A_1ax_2 - A_2ax_1 / ay_1ax_2 - ay_2ax_1$$

Where,

C_x = Concentration of Cilostazol

C_y = Concentration of Zonisamide

A_1 = Absorbance of test at λ_1 (λ_{max} of CIL= 257.60 nm)

A_2 = Absorbance of test at λ_2 (λ_{max} of ZON= 283.80 nm)

ax_1 = Absorptivity of x drug (ZON) at λ_1

ax_2= Absorptivity of x drug (ZON) at λ_2

ay_1 = Absorptivity of y drug (CIL) at λ_1

ay_2= Absorptivity of y drug (CIL) at λ_2

SPECTROPHOTOMETRIC CONDITION

Table: 1.3 Spectrophotometric conditions for Spectroscopic Method

Mode	Spectrum
Scan Speed	Medium
Wavelength Range	400-200 nm
Initial base line correction	Methanol

PREPARATION OF CALIBRATION CURVE

Calibration Curve for Zonisamide: This series consisted of five concentrations of standard ZON solution ranging from 4-20µg/ml. The solutions were prepared by pipette out Standard ZON stock solution (0.4ml, 0.8ml, 1.2ml, 1.4ml and 2.0ml) was transferred into a series of 10 ml volumetric flask and volume was adjusted up to mark with Methanol. A zero order spectrum, measured the absorbance at 283.80nm against a reagent blank solution (Methanol).

Calibration Curve for Cilostazol: This series consisted of five concentrations of standard CIL solution ranging from 4-20µg/ml. The solutions were prepared by pipette out Standard CIL stock solution (0.4ml, 0.8ml, 1.2ml, 1.4ml and 2.0ml) was transferred into a series of 10 ml volumetric flask and volume was adjusted up to mark with Methanol. A zero order spectrum measured the absorbance at 257.60nm against a reagent blank solution (Methanol).

VALIDATION OF PROPOSED METHOD[17]

LINEARITY AND RANGE

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of **4-20µg/ml** and **4-20µg/ml** for **ZON** and **CIL** respectively **(n=6).**

PRECISION

Intraday Precision: The precision of the developed method was assessed by analyzing combined standard solution containing three different concentrations 8, 12, 16µg/ml for ZON and 8, 12, 16µg/ml CIL. Three replicate (n=3) each on same day. These% RSD value was found to be less than 1.0 indicated that the method is precise.

Interday Precision: The precision of the developed method was assessed by analyzing combined standard solution containing three different concentrations 8, 12, 16µg/ml for ZON and 8, 12, 16µg/ml CIL triplicate (n=3) per day for consecutive 3 days for inter-day precision. These %RSD value was found to be less than 2.0 indicated that the method is precise.

ACCURACY

The accuracy of the method was established using recovery technique i.e. external standard addition method. The known amount of standard was added at three different levels to pre analyzed sample. Each determination was performed in triplicate. The accuracy of the method was checked by recovery experiment performed at three different levels of 80%, 100% and 120 %. Percentage recovery for Zonisamide and Cilostazol were calculated. From the Synthetic Mixture weigh accurately equivalent about 8mg of ZON. Take Four 100ml Volumetric Flask and in each flask add synthetic mixture equivalent to 8mg of ZON. Flask 1 form as a Placebo and remaining flask 2, 3 and 4 spike with 80, 100 and 120%. Same way spike CIL in respectively flasks and dissolved in 25 ml Methanol and Sonicated for 15min. make up the volume with methanol. The solution was filtered through Whatman filter paper No. 42. Finally the solution had concentration 80µg/ml for ZON and 80µg/ml for CIL. From that pipette out 0.8 ml in 10 ml volumetric flask and volume was made up to mark with Methanol to make final concentration ZON (8µg/ml) and CIL (8µg/ml). Data from nine determinations over three concentration levels covering the specified range was determined and % recovery was calculated. Recovery between 98-102 % justifies the accuracy of the method.

LOD and LOQ

The Limit of detection and quantitation of the developed method was assessed by analysing 10 replicates of standard solutions containing concentrations 4µg/ml for ZON and 4µg/ml for CIL.

LOD: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD was calculated out by using following formula:

$DL = 3.3 \sigma / S$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

LOQ: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

LOQ was calculated out by using following formula:

$DL = 10 \sigma / S$

ROBUSTNESS

Robustness of the method was determined by subjecting the method to slight change in the method condition, individually,

- Change Analyst
- % RSD was calculated.

RUGGEDNESS

Ruggedness of the method was determined by subjecting the method to slight change in the method condition, individually, the:

- Change in Wavelength from 283.80 nm and 257.60 nm to 283±2 nm and 257±2 nm.
- Three replicates were made for the same concentration
- % RSD was calculated.

ASSAY BY UV SPECTROPHOTOMETRIC METHOD

- The preparation of synthetic mixture as per patent:
  - Zonisamide: 100 mg
  - Cilostazol: 100 mg
  - Magnesium Stearate: 40 mg
  - Talc : 950 q.s
- From the Synthesis Mixture weigh accurately equivalent about 10mg of ZON (10 mg of CIL) in 100ml Volumetric Flask and dissolve in 25ml of Methanol. The flask was sonicated for 15 min. Dilute up to the 100 ml with Solvent. Shake vigorously; filter the solution and further Dilution.
- Finally the solution had the concentration 100µg/ml and 100µg/ml respectively for ZON and CIL. After that from this solution 1.2ml was pipette out and diluted up to 10 ml with Methanol. So the concentration was 12µg/ml and 12µg/ml for ZON and CIL respectively.

RESULT AND DISCUSSION

The methods were validated with respect ICH Q_2R_1 guidelines.

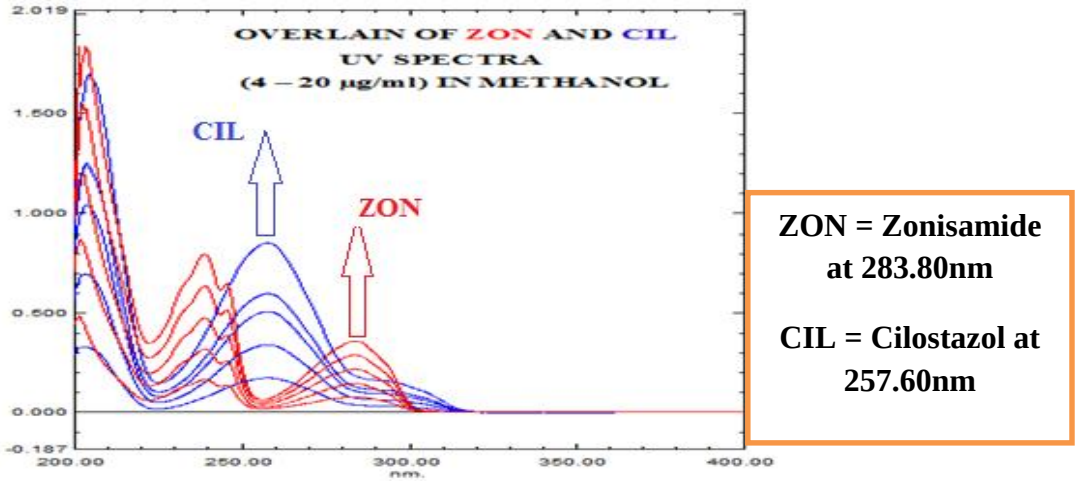

Figure 1.1: Overlain Spectra of ZON and CIL (4 – 20 µg/ml) in Methanol

Validation of Proposed Spectrophotometric Method for Simultaneous Method

LINEARITY AND RANGE

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of **4-20µg/ml** for **ZON** and **CIL** respectively **(n=6).**

LINEARITY OF ZONISAMIDE

Table 1.4: Linearity of Zonisamide

Sr. No.	ZON µg/ml	Absorbance ± SD 283.80nm (n=6)	%RSD	ZON µg/ml	Absorbance ± SD 257.60nm (n=6)	%RSD
1.	4	0.076 ± 0.0004	0.538	4	0.017 ± 0.00004	0.004
2.	8	0.142 ± 0.0006	0.445	8	0.025 ± 0.0008	1.622
3.	12	0.215 ± 0.0006	0.294	12	0.039 ± 0.0004	1.051
4.	16	0.286 ± 0.0004	0.143	16	0.052 ± 0.0004	0.788
5.	20	0.357 ± 0.0008	0.229	20	0.065 ± 0.0004	0.630

LINEARITY OF CILOSTAZOL

Table 1.5: Linearity of Cilostazol

Sr. No.	CIL µg/ml	Absorbance ± SD 257.60nm (n=6)	%RSD	CIL µg/ml	Absorbance ± SD 283.80nm (n=6)	%RSD
1.	4	0.171 ± 0.0004	0.239	4	0.036 ± 0.0005	1.450
2.	8	0.337 ± 0.0008	0.188	8	0.072 ± 0.0008	1.170
3.	12	0.505 ± 0.0004	0.081	12	0.105 ± 0.0005	1.150
4.	16	0.690 ± 0.0005	0.075	16	0.145 ± 0.0004	0.281
5.	20	0.850 ± 0.0004	0.048	20	0.183 ± 0.0005	0.283

LINEARITY OF MIXTURE

Table 1.6: Linearity of Mixture

Sr. No.	ZON µg/ml	Absorbance ± SD 283.80nm (n=6)	%RSD	CIL µg/ml	Absorbance ± SD 257.60nm (n=6)	%RSD
1.	4	0.109 ± 0.0006	0.580	4	0.185 ± 0.0006	0.342
2.	8	0.218 ± 0.0004	0.187	8	0.376 ± 0.0006	0.168
3.	12	0.325 ± 0.0005	0.159	12	0.562 ± 0.0004	0.073
4.	16	0.436 ± 0.0004	0.094	16	0.752 ± 0.0006	0.084
5.	20	0.552 ± 0.0004	0.740	20	0.948 ± 0.0006	0.067

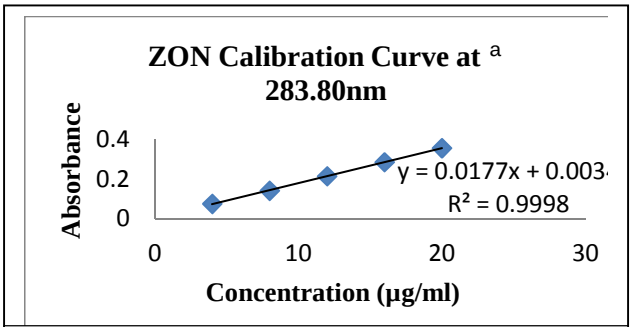

Fig. 1.2: Linearity of ZON Calibration Curve at 283.80nm

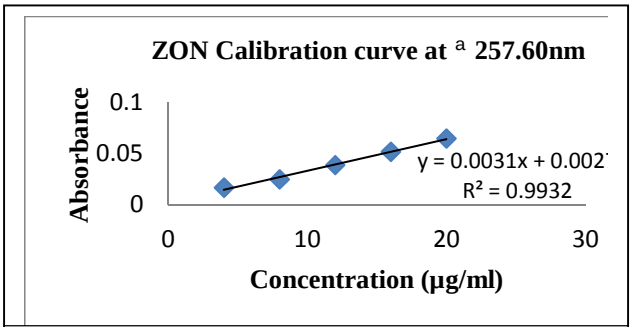

Fig. 1.3: Linearity of ZON Calibration Curve at 257.60nm

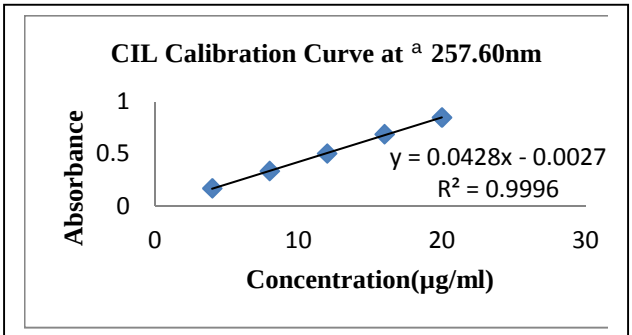

Fig. 1.4: Linearity of CIL Calibration Curve at 257.60nm

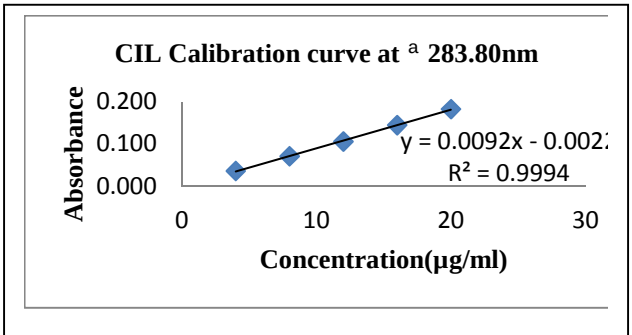

Fig. 1.5: Linearity ofCIL Calibration Curve at 283.80nm

PRECISION

INTRADAY PRECISION

- % RSD was found to 0.133 to 0.308 % for Zonisamide and 0.077 to 0.178 % for Cilostazol.

Table 1.7: Intraday Precision data for estimation of ZON and CIL (n=3)

Conc. (µg/ml)	Zonisamide and Cilostazol			
ZON+CIL	Absorbance 283.80nm	% RSD	Absorbance 257.60nm	% RSD
8:8	0.218 ± 0.0006	0.265	0.377 ± 0.0006	0.153
12:12	0.325 ± 0.0010	0.308	0.563 ± 0.0010	0.178
16:16	0.436 ± 0.0006	0.133	0.752 ± 0.0006	0.077

INTERDAY PRECISION

- % RSD was found to 0.178 to 0.459 % for Zonisamide and 0.103 to 0.307 % for Cilostazol.

Table 1.8: Interday Precision data for estimation of ZON and CIL (n=3)

Conc. (µg/ml)	Zonisamide and Cilostazol			
ZON+CIL	Absorbance 283.80nm	% RSD	Absorbance 257.60nm	% RSD
8:8	0.218 ± 0.0010	0.459	0.377 ± 0.0012	0.307
12:12	0.325 ± 0.0006	0.178	0.563 ± 0.0006	0.103
16:16	0.437 ± 0.0010	0.229	0.753 ± 0.0010	0.133

ACCURACY

Accuracy of the method was confirmed by recovery study from Synthetic mixture at three levels. The % recovery was found within 100.62 to 101.38 % and 101.48 and 101.76% for Zonisamide and Cilostazol respectively.

Table 1.9: Accuracy of ZON and CIL

Amt. of Formulation(mg)		Amt. of API Spiking (mg)		Total Amt. (mg)	
ZON	CIL	ZON	CIL	ZON	CIL
8	8	-	-	8	8
8	8	7	7	15	15
8	8	8	8	16	16
8	8	9	9	17	17

Table 1.10: % Recovery data of ZON and CIL (n=3)

% Recovery	Total Con.		Amplitude		Con. Found (8mg)		% Recovery ± SD		% RSD	
Drug	ZON	CIL	ZON	CIL	ZON	CIL	ZON	CIL	ZON	CIL
Control	8	8	-	-	-	-	-	-	-	-
80 %	15	15	0.413	0.714	15.09	15.11	101.38 ± 0.329	101.76 ± 0.165	0.324	0.162
			0.412	0.714						
			0.413	0.713						
100 %	16	16	0.439	0.762	16.05	16.13	100.62 ± 0.500	101.54 ± 0.144	0.496	0.142
			0.440	0.761						
			0.438	0.762						
120 %	17	17	0.468	0.810	17.09	17.13	101.07 ± 0.256	101.48 ± 0.339	0.254	0.334
			0.468	0.811						
			0.467	0.808						

LOD and LOQ

Table 1.11: LOD & LOQ value of ZON and CIL (n=10)

Drugs		LOD (µg/ml)	LOQ (µg/ml)
ZON + CIL	283.80nm	0.0566	0.1714
	257.60nm	0.0394	0.1193

ROBUSTNESS

Table 1.12: Different Analyst (n=3)

Parameter		ZON 283.80nm	% RSD	CIL 257.60nm	% RSD
Different Analyst	**Analyst-1**	0.106 ± 0.0005	0.546	0.184 ± 0.0005	0.314
	Analyst-2	0.106 ± 0.0010	0.943	0.184 ± 0.0010	0.543

RUGGEDNESS

Table 1.13: Change in Wavelength of 283.60 nm (n=3)

Parameter	Wavelength	ZON 283.80nm	% RSD
Change in Wavelength	281.80	0.106 ± 0.0006	0.546
	285.80	0.106 ± 0.0005	0.543

Table 1.14: Change in Wavelength of at 257.60nm (n=3)

Parameter	Wavelength	CIL 257.60nm	% RSD
Change in Wavelength	255.60	0.183 ± 0.0006	0.316
	259.60	0.183 ± 0.0005	0.315

ASSAY BY UV SPECTROPHOTOMETRIC METHOD FOR SIMULTENOUS METHOD

Table 1.15: Assay (n=3)

Drugs		% Assay ± SD	% RSD
ZON + CIL	283.80nm	100.69 ± 0.177	0.176
	257.60nm	100.05 ± 0.272	0.272

Table 1.16: Summary of Validation Parameter

Sr. No.	Parameter	Zonisamide	Cilostazol	Acceptance Criteria
1.	Linearity & Range(n=6)	4-20 µg/ml	4-20 µg/ml	-
2.	Regression Equation	y= 0.0275x-0.0024	y= 0.0476x-0.006	-
3.	Correlation Coefficient (r^2)	0.9999	0.9999	NLT 1
4.	Precision (n=3)			% RSD NMT 2.0 %
	A. Intraday	0.113 - 0.308	0.077 - 0.178	
	B. Interday	0.178 - 0.459	0.103 - 0.307	
5.	Accuracy (% Recovery) (n=3)	100.62 to 101.38 %	101.48 to 101.76%	NLT 98.0 % to NMT 102.0 %
6.	LOD (n=10)	0.0566 µg/ml	0.0394 µg/ml	-
7.	LOQ(n=10)	0.1714 µg/ml	0.1193 µg/ml	-
8.	Robustness (n=3)	0.546-0.943	0.314-0.543	% RSD NMT 2.0 %
9.	Ruggedness(n=3)	0.543-0.546	0.315-0.316	% RSD NMT 2.0 %
10.	Assay(n=3)	100.69 ± 0.177	100.05 0.272	-

CONCLUSION

A simple, specific, accurate and precise Zero order derivative spectrophotometric method was developed and validated for simultaneous estimation of Zonisamide and Cilostazol in Synthetic Mixture. The wavelength of estimation for Zonisamide was 283.80 nm and for Cilostazol was 257.60 nm. Beer's law is obeyed in the concentration range of 4-20µg/ml and 4-20µg/ml and correlation coefficient of 0.9999 and 0.9999 for Zonisamide and Cilostazol respectively. The % recovery for Zonisamide and Cilostazol were found to be 100.62 % - 101.32 % and 101.48 - 101.76 % respectively. Intraday precision of Zonisamide and Cilostazol were found to be 0.113 – 0.308 and 0.077 – 0.178 % RSD and Interday precision were found to be 0.178 – 0.459 and 0.103 – 0.307 % RSD respectively. The proposed method was also evaluated by the Assay of Synthetic mixture containing Zonisamide and Cilostazol. The % Assay was found to be 100.69 ±

0.177 for Zonisamide and 100.05 ± 0.272 for Cilostazol. The method is validated as per ICH Q_2R_1Guidelines.

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