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DEVELOPMENT AND VALIDATION OF SOME CLASSICAL AND NOVEL SPECTROPHOTOMETRIC METHODS AND RP-HPLC METHOD BY QbD APPROACH FOR SIMULTANEOUS ESTIMATION OF ARIPIPRAZOLE AND CLOZAPINE IN SYNTHETIC MIXTURE

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ABSTRACT

The present study portrays a simple, rapid, sensitive, accurate, precise and cost effective UV Spectrophotometric methods and RP-HPLC method for the simultaneous estimation of Aripiprazole and Clozapine in Synthetic Mixture. UV Spectrophotometric methods applied are: 1) First Derivative Zero Crossing Point Method 2) Absorption factor method 3) Chemometrics assisted UV spectrophotometric methods. The RP-HPLC method applied by QbD approach. Proposed techniques have been validated as per ICH guideline and successfully applied to the simultaneous estimation of Aripiprazole and Clozapine in their Synthetic Mixture. The results of analysis have been validated statistically and by recovery studies.

KEYWORDS: Aripiprazole (Ari), Clozapine (Clz), classical and novel UV spectrophotometric methods, RP-HPLC methods by QbD approach

INTRODUCTION

Aripiprazole (Ari) and Clozapine (Clz) is an Atypical Antipsychotic drug. Chemically Aripiprazole is 7-{4-[4-(2, 3- chlorophenyl) piperazin-1-yl] butoxyl}-1,2,3,4-tetrahydroquinolin-2-one. It is primarily used in the schizophrenia and bipolar disorder. Although it is used as an add-on treatment in major depressive disorder, psychotic disorders, and irritability associated with autism. Aripiprazole have partial agonistic activity at D2 receptor, also have partial agonist activity at 5-HT1A receptor, and have antagonist activity at

5-HT2A receptor. Clozapine is chemically 8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine. It works by changing the activities of chemicals in the cerebrum.[1] It is used to treat severe schizophrenia, or to reduce the risk of suicidal behavior in people with schizophrenia or similar disorders. It is also used in Parkinson's disease.[2]

Combination of Aripiprazole and Clozapine was studied under clinical trial phase and was proved that the synergistic effect was observed by improving psychotic symptoms and reducing side effects such as agranulocytosis, sedation, weight gain, sialorrhoea, and enuresis as compare to Clozapine monotherapy. Also combination therapy leads to dose reduction of Clozapine. Although combination of Aripiprazole and Clozapine leads to improve in positive and negative symptoms. Also significant improvement in mean BPRS score (brief psychiatric rating scale) in combination therapy. There is higher metabolic risk in Clozapine monotherapy due to its strong blockade of 5HT2C and Histamine H1 receptors and stimulation of hypothalamic AMPK (adenosine monophosphate activated protein kinase), an enzyme that reverses the effect of leptin. Unlike Clozapine, Aripiprazole has no histaminergic activity, and is a 5HT2C agonist. Moreover, it has some agonist activity at 5HT1A receptors, believed to lower blood glucose levels. Therefore, there is a mechanistic reasoning behind augmenting Clozapine with Aripiprazole- the effect of Aripiprazole on 5HT2C and 5HT1A receptors may in fact protect against the diabetes, weight gain and dyslipidaemia induced by Clozapine.[1] Marketed formulation is not available and the analytical study was carried out in synthetic mixture.

EXPERIMENTAL SECTION

Whole experiment is divided in three sub-parts:

1) Classical and novel UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine
2) Chemometrics assisted UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine
3) Development and Validation of both drugs in combination by QbD approach for isocratic RP-HPLC method.

Instruments

1) *UV-VIS spectrophotometer*, UV-1700 pharmaspec model, from Shimadzu, Japan. This spectrophotometer is adequate and equipped with quartz cuvettes having the optical path of 10 mm.

2) HPLC, SHIMADZU LC-20AT Prominence

Column: WATERS C-18 column (250 mm x 4.6 mm, 5 µm)

Detector: SHIMADZU SPD-20A Prominence UV/VIS Detector

Injector: Rheodyne 7725 injector valve with fixed loop at 20µl.

Material and Reagents: Aripiprazole and Clozapine API and tablets.

Selection of a Solvent

Both The Drugs were soluble in Methanol. So, Methanol was selected as a solvent for estimation of both the Drugs.

1) Classical and novel UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine

Preparation of Standard stock and working standard solution

Preparation of standard stock solution of Aripiprazole (100 µg\ml): Weighed accurately 10 mg of Aripiprazole and was transferred into 100 ml volumetric flask, diluted to half and sonicated and made up to the mark with Methanol. (100 µg/ml).

Preparation of working standard solution of Aripiprazole (10 µg\ml): Withdraw 1 ml from the stock solution and transferred into 10 ml volumetric flask and diluted with Methanol to obtain 10 µg/ml.

Preparation of standard stock solution of Clozapine (1000 µg\ml): Weighed accurately 10 mg of Clozapine and was transferred into 10 ml volumetric flask, diluted to half and sonicated and made up to the mark with Methanol. (1000 µg/ml).

Preparation of working standard solution of Clozapine (100 µg\ml): Pipetted out 1 ml from the stock solution and transferred into 10 ml volumetric flask and diluted with Methanol to obtain 100µg/ml.

Method A: Classical - First Derivative Zero Crossing Point Method

Derivative spectra can be used to enhance differences among spectra, to resolve overlapping bands in qualitative analysis and most importantly, to reduce the effects of interference from scattering, matrix or other absorbing compounds in quantitative analysis.

Procedure of selection of wavelength

0.2 ml working standard solution of Aripiprazole (10 µg/ml) and 0.2 ml working standard solution of Clozapine (100 µg/ml) was transferred into different 10 ml volumetric flask and dilute up to mark with Methanol to get 0.2 µg/ml of Aripiprazole and 2.0 µg/ml of Clozapine. Each solution was scanned in the range of 200-400 nm. Zero Order spectra were converted into First Order spectra. Aripiprazole shows ZCP (Zero Crossing Point) at 239.50 nm and Clozapine show ZCP at 225.50 nm(Figure 1). Hence, these wavelengths 225.50 and 239.50 were selected as analytical wavelengths for Aripiprazole and Clozapine respectively.

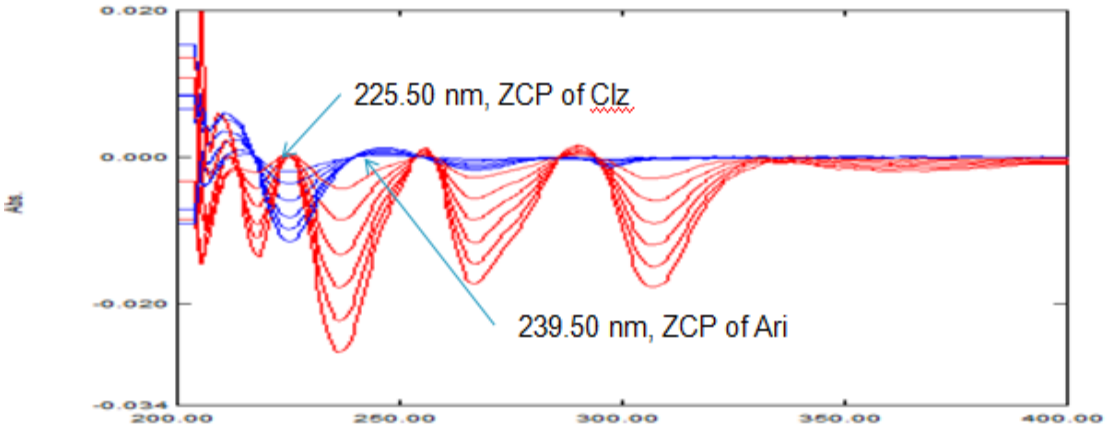

Figure 1: Overlain spectra of Calibration curve.

Method B: Novel – Absorption factor method

Quantitative estimation of Ari was carried out by subtracting interference of Clz using experimentally calculated absorption factor.

Procedure of selection of wavelength

0.2 ml working standard solution of Aripiprazole (10 µg/ml) and 0.2 ml working standard solution of Clozapine (100 µg/ml) was transferred into different 10 ml volumetric flask and dilute up to mark with Methanol to get 0.2 µg/ml of Aripiprazole and 2.0 µg/ml of Clozapine. Each solution was scanned in the range of 200-400 nm (Figure 2). Hence, 217.50 and 311.0 were selected as analytical wavelengths for Aripiprazole and Clozapine respectively. The value of Absorption factor was found to be 1.1264. Quantitative estimation of the Ari and Clz was carried out using following equation.

Absorption of Ari at 217.5 nm =

Abs 217.5 (Ari+Clz) – [Abs217.5 (Clz)/Abs 311 (Clz)] X Abs311 (Ari+Clz)

Abs: Absorption Value

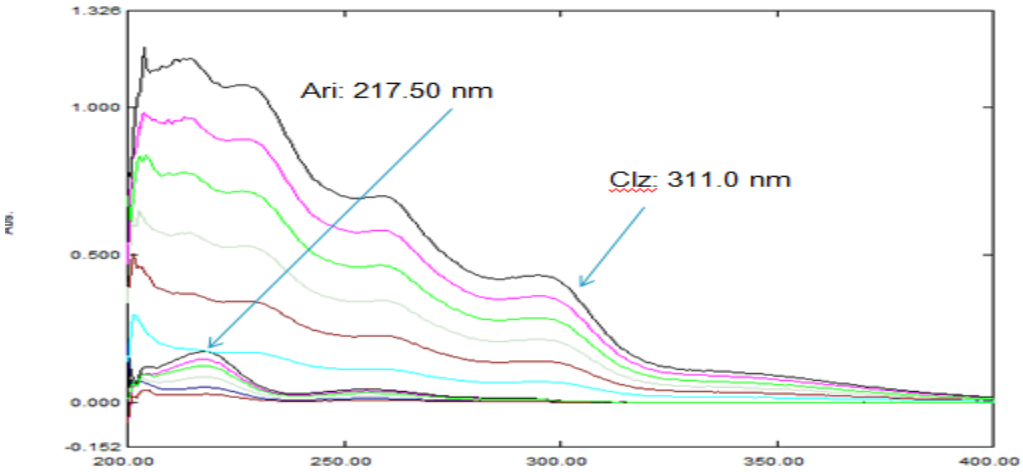

Figure 2: Overlay spectra of Calibration curve.

***Method Validation*[3,4]**

Developed spectrophotometric methods for the simultaneous estimation Of Ari and Clz were validated according to ICH Q2 (R1) guidelines and data complying with the standards were obtained. The proposed technique has been extensively validated in terms of linearity, accuracy and precision, limit of detection and limit of quantification.

2) Chemometrics assisted UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine

- Chemometric methods are one kind of multivariate analysis in which multiple measurements are made on a sample of interest. So, more than one variable or response are measured for each sample.

  The chemometric quantitative analytical techniques have many applications and advantages such as:

- ✓ Allow the resolution of the complex spectra of mixtures of analytes.
- ✓ The mixtures can be analyzed without any separation procedures for drug determination.
- ✓ The techniques are very easy to apply, very sensitive, useful and yet very inexpensive as compared to other analytical techniques for simultaneous determination of compounds in multicomponent mixtures.
- ✓ Allow the interpretation of multivariate data and is vital to the success of the simultaneous determination of the organic components.

- Multivariate methods include two type of methods

I) Multiple linear regression (MLR) methods:

a) *Classical Least Squares (CLS) or (K-matrix)*

b) Inverse Least Squares (ILS) or (P-matrix)

II) Factor-based methods:

a) *Principal Component Regression (PCR)*

b) Partial Least Squares (PLS)

- **Preparation of Binary Mixtures (Ari + Clz) for Calibration set and Validation set :**

➢ Appropriate and accurate volume aliquots of the stock solutions were taken according to following Binary mixture scheme and volume made up to 10ml with water.

➢ The absorbance data matrix was obtained by measuring the absorbance at each wavelength points (210 to 400nm) with the interval of 5nm (delta lambda 5nm) in spectral region between 210 to 400 nm.

➢ 36 sets are taken for Calibration set and for validation set.

- Calibration Set.
- A set of 25 mixtures was prepared in water, applying a multilevel multifactor design in which two levels of concentrations of Ari and Clz within the stated range were introduced as shown in Table 1.

Table 1: Calibration Set

No. of mixtures	conc. (µg/ml) Ari + Clz	No. of mixtures	conc. (µg/ml) Ari + Clz	No. of mixtures	conc. (µg/ml) Ari + Clz
1	0.2+6	10	1.0+8	19	0.8+12
2	1.2+10	11	0.4+2	20	1.2+2
3	1.2+4	12	0.8+2	21	1.0+4
4	0.4+6	13	0.4+4	22	0.8+6
5	1.2+12	14	0.4+8	23	0.2+2
6	0.8+10	15	1.0+10	24	0.6+4
7	1.0+12	16	0.4+10	25	0.6+8
8	0.2+12	17	0.8+4		
9	1.2+8	18	0.6+6		

- Validation Set

A set of 11 mixtures was prepared in water, applying a multilevel multifactor design in which two levels of concentrations of Ari and Clz within the stated range were introduced as shown in Table 2.

Table 2: Validation Set					
No. of mixtures	conc. (µg/ml) Ari + Clz	No. of mixtures	conc. (µg/ml) Ari + Clz	No. of mixtures	conc. (µg/ml) Ari + Clz
1	0.6+10	5	1.0+2	9	0.8+8
2	0.6+2	6	0.2+10	10	0.6+12
3	0.2+4	7	1.2+6	11	0.2+8
4	0.4+12	8	1.0+6		

1.Classical least squares (CLS)

- CLS is one of the simplest methods, based on a linear relationship between the absorbance and the component concentrations at each wavelength (Figure 3).

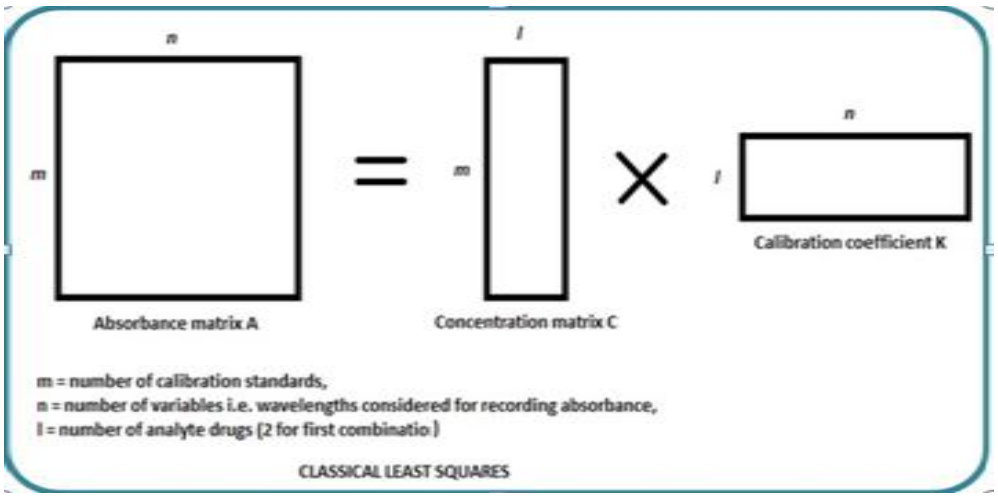

Figure 3: Classical least squares.

- In matrix notation, the Beer's law models for *m calibration standards containing l* chemical components with spectra of *n* digitized absorbance is given by.

$A=C*K$ …….. *(1)*

Where,

A is the *m x n matrix of calibration* spectra,

C is the m x l matrix of component concentrations,

K is the l x n matrix of absorbance-concentration proportionality constants (absorptivity- path length).

K = pinv (C) * A

- Once we have K, we can determine C for unknown samples from its absorbance matrix A

$C_{sample} = A_{sample} * pinv (K)$

2.Inverse least squares (ILS)

- This method treats concentration as a function of absorbance (Figure 4).

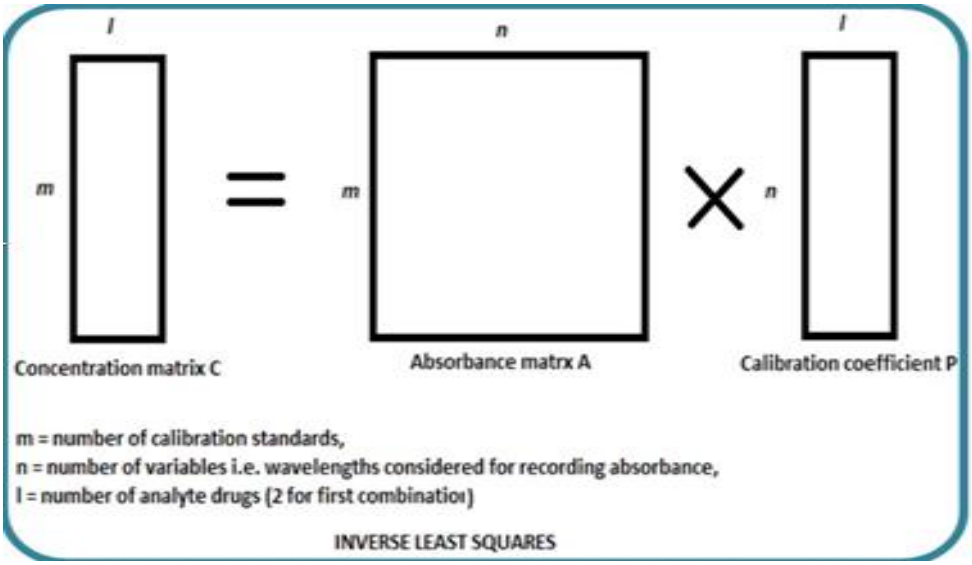

Figure 4: Inverse least squares.

- The inverse of Beer's law model for *m calibration standards with spectra* of *n digitised absorbance is given by.*

$C=A*P$ ……(2)

Where,

C and A are as before

P is the n × l matrix of unknown calibration co-efficient relating the *l component concentrations of the* spectral intensities.

P = pinv (A) * C

- Once we have P, we can determine C for unknown samples from its absorbance matrix A

$C_{sample} = A_{sample} * P$

3.Principal Component Regression (PCR).

- X, the predictors are absorbance values of 25 calibration standards at 39wavelengths and Y, concentrations of Ari and Clz in 25 calibration standards.
- Each calibration standard described by total 39 variables i.e. wavelengths. Each calibration standard can be represented as a point in 39 dimensional space. Here, dimension means the vector representing absorbance at one particular wavelength, one vector for each of the wavelengths.

- What PCR does?: First step is Reducing the dimensionality of X space. PCR will compute a few principal components which can well describe the calibration standards so as to represent each sample by it's PCs instead of 39 variables (Principal Component Analysis). PCs are computed in a manner that first PC explains maximum variance of the data. Next step after Reducing the dimensionality is regression that PCs will then be correlated to Y space (Figure 5 and Figure 6).

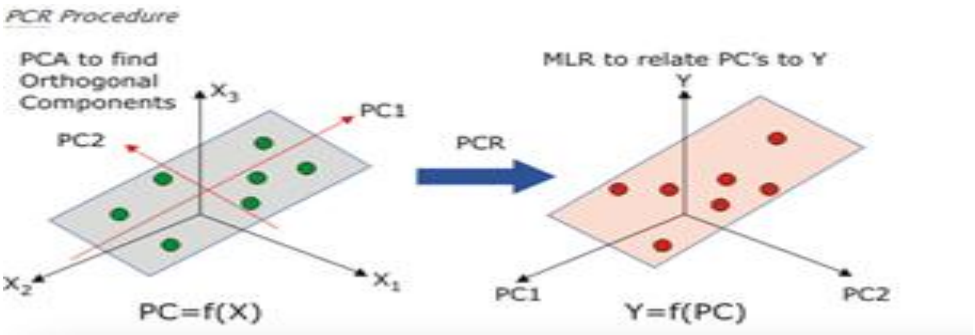

Figure 5

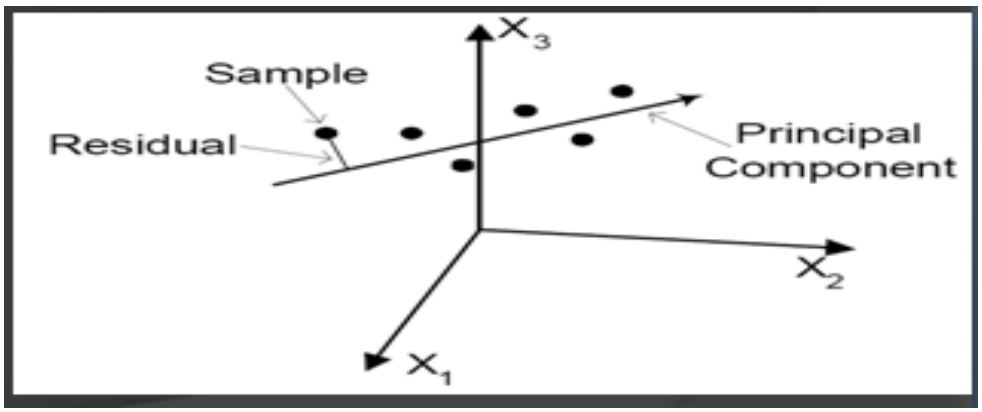

Figure 6.

4.Partial Least squares (PLS)

- Also known as 'Projection to Latent structures'
- An obvious extension of PCR
- Finding the latent variables in X that will best predict the latent variables in Y. these latent variables are similar to PCs and referred to as factors. In contrast to PCR, PCs are computed for both in PLS and then PC of X is related with PC of Y (Figure 7).

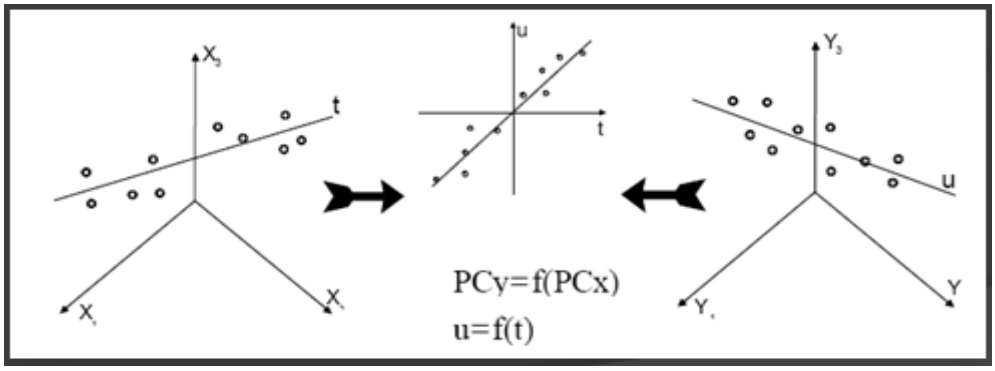

Figure 7.

Validation of all models carried out successfully.

3) Development and Validation of both drugs in combination by QbD approach for isocratic RP-HPLC method

Selection of Detection Wavelength: Standard solutions of Ari and Clz were scanned between 200-400 nm in UV-visible spectrophotometer and showed good sensitivity at 225 nm as shown in Figure 8 which was selected as the analytical wavelength.

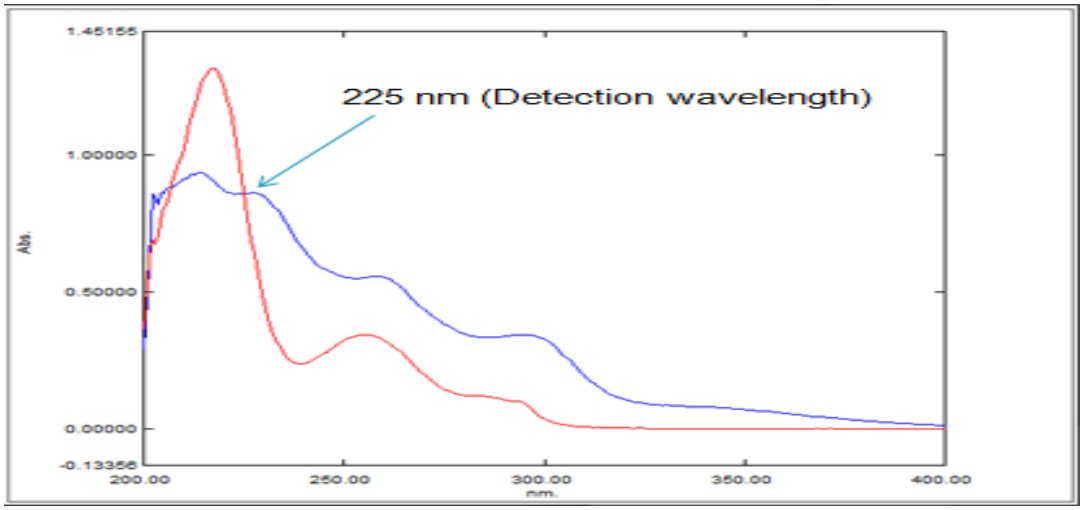

Figure 8: Overlain spectra of Ari and Clz.

What is QbD?[5,6]

- A QbD is a science and risk-based paradigm for analytical method development, endeavoring for understanding the predefined objectives to control the Critical Method Variables (CMVs) affecting the Critical Method Attributes (CMAs) to achieve enhanced method performance, high robustness, ruggedness and flexibility for continual improvement.

- A QbD helps in development of a robust and cost effective analytical method which is pertinent throughout the lifecycle of the product, to facilitate the regulatory flexibility in analytical.
- Critical method parameters(CMPs): "parameters whose variability have an impact on a CQA and therefore should be monitored or controlled to ensure the process produces the desired quality"
- Critical quality attribute (CQA): "physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality" (Table 3).

Table 3: CQAs		
1	Retention Time	Marker of the separation ability of compound
2	No. of plates	Indicator of the mobile phase suitability and method performance
3	Tailing factor	Indicator of method efficiency
4	Resolution	Quantitative measure of how well two peaks can be differentiated

Categorization of parameters

- CNX Approach (control, noise, experimental) (Table 4).
- REM(Risk estimation matrix) (Table 9).
- Initial trials (Table 10).

Table 4: CNX Approach	
Control variables	Column stationary phase, Organic modifier type, Buffer type, Wavelength
Noise variables	pH meter calibration, instrument calibration(detector, pump, injector, tubings), material purity
Experimental variables	pH of buffer, buffer ratio, injection volume, flow rate

Primary hazard analysis

Table 5: Critical quality attributes(CQAs) - TAILING FACTOR						
	Severity		**Probability**		**Risk priority number**	
	Ari	**Clz**	**Ari**	**Clz**	**Ari**	**Clz**
Buffer pH	2	1	3	3	6	3
Buffer type	2	1	2	2	4	2
% aqueous	1	1	3	3	3	3
Flow rate	1	1	3	3	3	3
Injection volume	1	1	1	1	1	1

Table 6: Critical quality attributes(CQAs) - THEORITICAL PLATES						
	Severity		Probability		Risk priority number	
	Ari	Clz	Ari	Clz	Ari	Clz
Buffer pH	3	2	2	2	6	4
Buffer type	2	2	2	2	4	4
% aqueous	2	2	3	3	6	6
Flow rate	2	3	1	1	2	3
Injection volume	1	1	3	3	3	3

Table 7: Critical quality attributes(CQAs) - RETENTION TIME						
	Severity		Probability		Risk priority number	
	Ari	Clz	Ari	Clz	Ari	Clz
Buffer pH	2	2	3	3	6	6
Buffer type	3	3	2	2	6	6
% aqueous	3	2	3	3	9	6
Flow rate	1	1	3	3	3	3
Injection volume	1	1	1	1	1	1

Table 8: Critical quality attributes(CQAs) - RESOLUTION						
	Severity		Probability		Risk priority number	
	Ari	Clz	Ari	Clz	Ari	Clz
Buffer pH	2	1	1	1	2	1
Buffer type	3	2	3	3	9	6
% aqueous	3	1	2	2	6	2
Flow rate	1	2	3	3	3	6
Injection volume	1	1	2	2	2	2

Risk estimation matrix

Table 9: Risk estimation matrix.								
	Tailing factor		Theoritical plates		Retention time		Resolution	
	Ari	Clz	Ari	Clz	Ari	Clz	Ari	Clz
Buffer pH								
Buffer type								
% aqueous								
Flow rate								
Injection volume								

Severe Moderate Low

Initial trials

Table 10: Initial trials.

Sr. No.	Mobile phase	% ratio	Retention time (min)		Comment
			Ari	Clz	
1	Methanol : Water	50 : 50	-	-	Peak overlapped
2	Acetonitrile : Water	50 : 50	-	-	Broad & splitted peak
3	Methanol : Phosphate buffer (pH-2.7)	50 : 50	14.8	7.1	Late elution
4	Methanol : Phosphate buffer (pH-2.7)	70 : 30	3.4	3.2	Too close peaks, no resolution
5	Methanol : Phosphate buffer (pH-2.7)	60 : 40	5.5	4.4	Low resolution (2.032 & 7.754)
6	Methanol : Phosphate buffer (pH-2.7)	55 : 45	8.2	5.6	Late elution
7	Methanol : Phosphate buffer (pH-3.0)	55 : 45	8.9	6.2	Late elution
8	Methanol : Phosphate buffer (pH-3.0)	60 : 40	3.5	3.3	Too close peaks, no resolution
9	Methanol : Phosphate buffer (pH-2.7)	58 : 42	6.9	5.1	Good resolution & good assymetry
10	Methanol : Formate buffer (pH-2.8)	50 : 50	14	7.4	Late elution & splitted peaks
11	Acetonitrile : Formate buffer (pH-2.8)	50 : 50	-	-	Broad & splitted peak
12	Acetonitrile : Formate buffer (pH-3.8)	50 : 50	-	-	Broad & splitted peak
13	Acetonitrile : Acetate buffer (pH-4.5)	50 : 50	5	3.8	Low resolution (3.211 & 0.754)
14	Acetonitrile : Methanol : Acetate buffer (pH-4.5)	40 : 20 : 40	6.1	4.4	Low resolution (1.799 & 6.908)
15	Methanol : Acetate buffer (pH-4.5)	50 : 50	16.9	15.1	Late elution
16	Acetonitrile : Methanol : Acetate buffer (pH-4.5)	40 : 10 : 50	9.4	5.9	Late elution
17	Methanol : Acetate buffer (pH-4.5)	70 : 30	9.2	5.8	Late elution
18	Acetonitrile : Methanol : Acetate buffer (pH-4.5)	30 : 20 : 50	12.4	7.2	Late elution
19	Acetonitrile : Methanol : Acetate buffer (pH-4.5)	30 : 30 : 40	10.3	6.5	Late elution
20	Acetonitrile : Methanol : Acetate buffer (pH-4.0)	40 : 20 : 40	5.04	4.06	Low resolution (1.803 & 4.376)
21	Acetonitrile : Methanol : Acetate buffer (pH-4.0)	30 : 40 : 30	4.3	3.8	Too close peaks, low resolution
22	Acetonitrile : Methanol : Acetate buffer (pH-4.0)	30 : 35 : 35	4.9	4.1	Too close peaks, low resolution
23	Acetonitrile : Methanol : Acetate buffer (pH-4.0)	30 : 30 : 40	5.8	4.5	Too close peaks, low resolution
24	Acetonitrile : Methanol : Acetate buffer (pH-4.3)	30 : 30 : 40	7.2	5.2	High tailing factor
25	Acetonitrile : Methanol : Acetate buffer (pH-4.3)	40 : 10 : 50	7.3	5	High tailing factor
26	Acetonitrile : Methanol : Acetate buffer (pH-4.3)	35 : 13 : 52	8.1	5.4	Good resolution & good assymetry

Note: Resolution of both drugs taken in consideration due to one unknown peak. Hence two resolution obtained.

Parameter screened

- Buffer type
- Buffer pH
- % Aqueous
- Injection volume
- Flow rate

Screening, optimization and validation of both drugs carried out successfully based upon the above data.

RESULT AND DISCUSSION

1) Classical and novel UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine

Method Validation

1. LINEARITY AND SENSITIVITY

The linearity of method was evaluated thrice by analyzing six concentration of each drug (Figure 9 and Figure 10). Linear regression equation was obtained over the concentration range (y = mx+c). Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from standard deviation of response and slope of calibration curve. Table 11 and 12 reveal the Summary of Validation parameters of Ari and Clz.

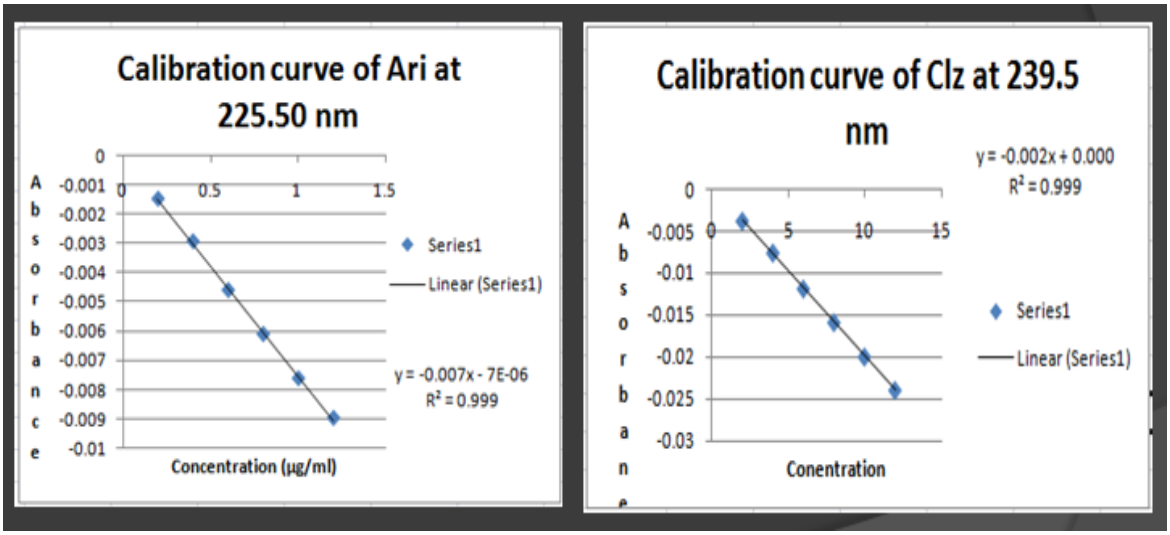

Figure 9: Calibration curve for Method A-First derivative ZCP method.

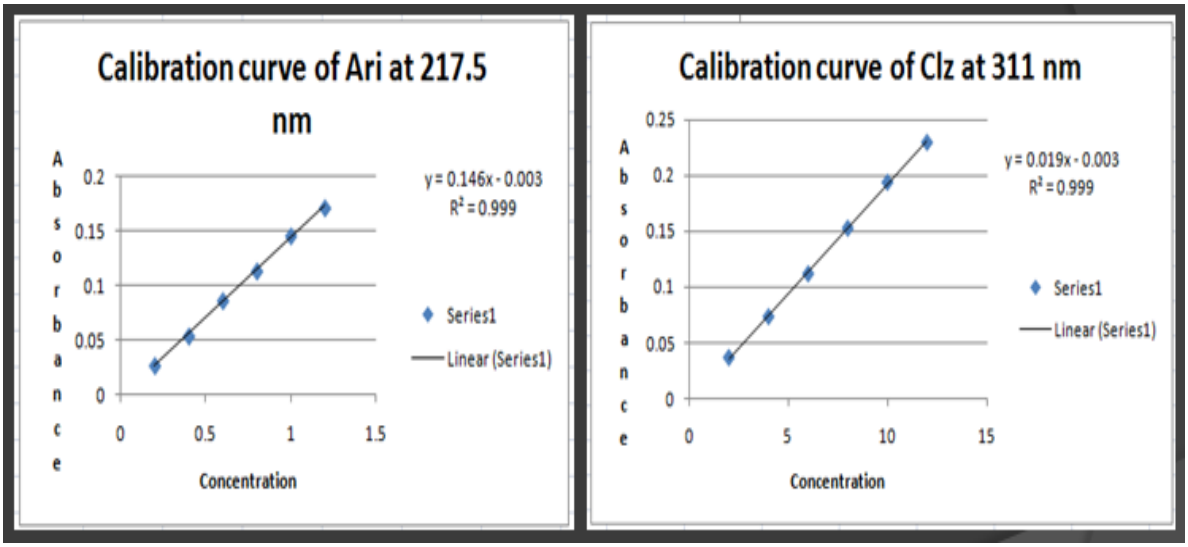

Figure 10: Calibration curve for Method B-Absorption factor method.

Table 11		
PARAMETERS	**First Derivative ZCP method(A)**	
	Ari	**Clz**
ANALYTICAL WAVELENGTH (nm)	225.50 nm	239.50 nm
BEERS'S RANGE(µg/ml)	0.2 – 1.2 µg/ml	2 - 12 µg/ml
SLOPE	-0.007	-0.002
INTERCEPT	7×10^{-6}	0.000
CORRELATION COEFFICIENT	0.999	0.999
STANDARD DEVIATION OF INTERCEPT	0.000	0.000
LIMIT OF DETECTION (LOD)	3.3×10^{-4} µg/ml	0.001 µg/ml
LIMIT OF QUANTIFICATION (LOQ)	1×10^{-3} µg/ml	0.001 µg/ml

Table 12		
PARAMETERS	**Absorption factor method(B)**	
	Ari	**Clz**
ANALYTICAL WAVELENGTH (nm)	217.50 nm	311.0 nm
BEERS'S RANGE(µg/ml)	0.2 – 1.2 µg/ml	2 - 12 µg/ml
SLOPE	0.146	0.019
INTERCEPT	-0.003	-0.003
CORRELATION COEFFICIENT	0.999	0.999
STANDARD DEVIATION OF INTERCEPT	0.000	0.000
LIMIT OF DETECTION (LOD)	0.056 µg/ml	0.18 µg/ml
LIMIT OF QUANTIFICATION (LOQ)	0.17 µg/ml	0.56 µg/ml

2. PRECISION

Intraday and Interday precision was measured in terms of % RSD. The experiment was repeated 3 times a day for intra day and for 3 different days for inter-day precision. The average % RSD was found to be less than 2.0% for both the methods (Table 13).

Table 13: Precision				
PARAMETER	**First derivative ZCP method(A)**		**Absorption factor method (B)**	
	Ari	**Clz**	**Ari**	**Clz**
INTRADAY PRECISION (%RSD)	0.29	0.14	0.06	0.11
INTERDAY PRECISION (%RSD)	0.25	0.19	0.12	0.30

3 ROBUSTNESS

- It should show the reliability of an analysis with respect to deliberate variations in method parameters as shown in Table 14.
- If measurements are susceptible to variation in analytical conditions, the analytical condition should be suitably controlled or a precautionary statement should be included in the procedure.

Table 14: Robustness				
PARAMETER : Methanol mfg	**First derivative ZCP method(A)**		**Absorption factor method (B)**	
	Ari	**Clz**	**Ari**	**Clz**
Rankem	0.22	0.18	0.67	0.19
Fischer	0.16	0.33	0.23	0.11
Spectrochem	0.17	0.33	0.16	0.32

4 RUGGEDNESS

Ruggedness means the ability of an analytical method to remain unaffected by small variations in method parameters and influential environmental factors and characterize its reliability during normal usage as shown in Table 15.

Table 15: Ruggedness				
Parameters: Two different analysts	**Ari**		**Clz**	
	Analyst A	**Analyst B**	**Analyst A**	**Analyst B**
%RSD	0.675	0.786	0.546	1.012

5 ACCURACY

To check the Accuracy of different methods, Recovery studies were carried out from pre-analyzed sample at three deferent level of standard addition 80%, 100% and 120%. Results of Recovery studies are shown in Table 16. For each of the method explained above, %Recovery was the average of three determinations at each standard addition level. %Recovery for different methods was found to be between 98%-102% which prove that all the methods were accurate.

Table 16: Recovery.			
DRUG	**METHOD A**		
	% spiking	**Base Conc.**	**%RECOVERY ± SD**
Ari	80	0.5 µg/ml	100.08± 0.163
	100		98.21± 1.02
	120		99.37± 0.58
Clz	80	5 µg/ml	98.04 ±0.98
	100		100.6 ±0.12
	120		99.74 ±0.892
	Method B		
Ari	80	0.5 µg/ml	99.65% ± 0.819
	100		100.36% ± 0.921
	120		100.44% ± 1.174
Clz	80	5 µg/ml	100.02% ± 0.89
	100		99.16% ± 0.48
	120		98.55% ±0.93

Results of Simultaneous estimation of Ari and Clz in synthetic mixture by methods-A and B (Table 17).

Table 17: Assay		
SYNTHETIC MIXTURE :-LABEL CLAIM - Ari:Clz 0.5mg:5mg		
METHODS	**% ASSAY**	
	Ari	**Clz**
FIRST DERIVATIVE ZCP METHOD	98.65%	100.09%
ABSORPTION FACTOR METHOD	99.55%	100.95%

2) Chemometrics assisted UV spectrophotometric methods for simultaneous estimation of Aripiprazole and Clozapine

1.Classical least squares (CLS)

Table 18: Computed K- matrix.					
Wavelength	**Ari**	**Clz**	**Wavelength**	**Ari**	**Clz**
210	0.0105	0.0929	310	-0.0002	0.0201
215	0.0127	0.0958	315	-0.0002	0.0143
220	0.0123	0.0894	320	-0.0002	0.011
225	0.0082	0.0882	325	-0.0002	0.0095
230	0.0041	0.0873	330	-0.0002	0.0089
235	0.0023	0.0785	335	-0.0001	0.0085
240	0.002	0.0673	340	-0.0002	0.0083
245	0.0024	0.0602	345	-0.0001	0.0078
250	0.0029	0.0572	350	-0.0001	0.0072
255	0.0031	0.0568	355	-0.0001	0.0066
260	0.003	0.0571	360	-0.0001	0.0058

265	0.0024	0.0522	365	-0.0001	0.0051
270	0.0016	0.045	370	-0.0001	0.0043
275	0.0011	0.0396	375	-0.0001	0.0036
280	0.0009	0.0358	380	-0.0001	0.0029
285	0.0009	0.0343	385	-0.0001	0.0024
290	0.0008	0.0348	390	-0.0001	0.0019
295	0.0006	0.0355	395	-0.0001	0.0015
300	0.0001	0.0337	400	-0.0001	0.0011
305	-0.0001	0.0276			

2.Inverse least squares (ILS)

Table 19: Computed P- matrix.

Wavelength	Ari	Clz	Wavelength	Ari	Clz
210	-11.3121	-9.0832	310	9.5238	30.2901
215	25.0865	22.1387	315	28.9041	-41.7707
220	77.5679	-30.2905	320	26.1006	-27.0436
225	23.7578	-3.6541	325	-39.0378	-12.0981
230	-144.383	-1.2396	330	-118.4505	47.5351
235	-137.6891	-7.9095	335	-15.3437	-5.0056
240	-12.0976	-53.4283	340	-32.1359	3.4669
245	109.8133	53.2792	345	-89.4669	-47.061
250	-27.2602	28.0739	350	18.9939	-43.4099
255	-31.2809	48.5623	355	10.8733	38.572
260	-14.5925	-36.3553	360	-76.0714	-25.0168
265	151.2089	34.958	365	141.2019	4.1386
270	93.5937	5.6542	370	-18.9243	64.3823
275	-94.3885	-52.5155	375	-75.1133	-40.0764
280	-21.1136	16.7379	380	-11.099	-3.6447
285	112.6302	40.5928	385	-23.6453	84.7488
290	-16.6272	27.8108	390	9.0776	-85.636
295	42.2312	-30.9806	395	17.743	51.9418
300	15.404	4.2292	400	43.2701	-33.2194
305	-0.9642	-3.1621			

3.Principal Component Regression (PCR)

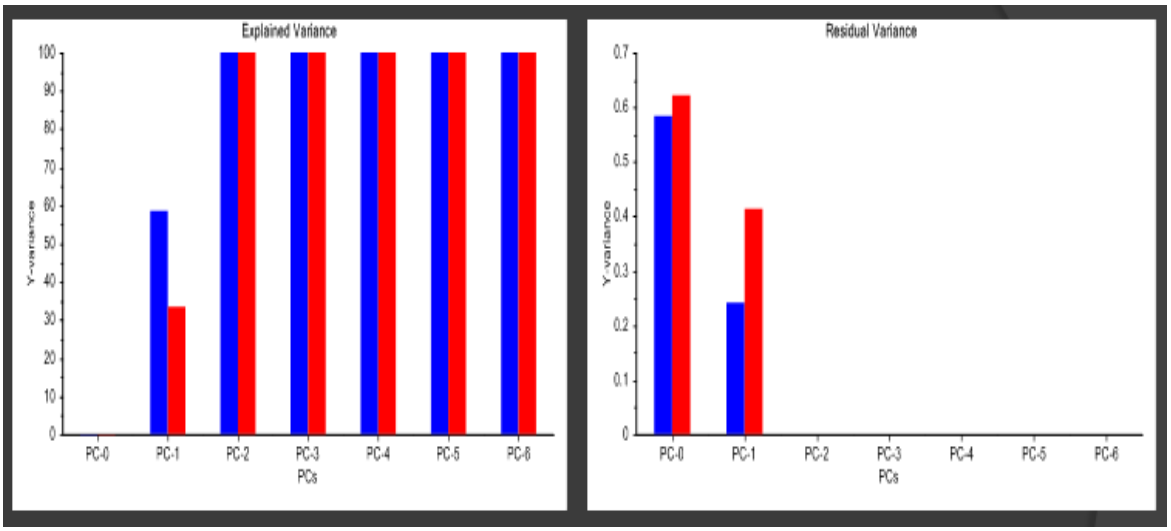

Figure 11

- Residual Variance – should be low (Near To Zero)
- Explained Variance – should be high (Near To 100)

4.Partial Least squares (PLS)

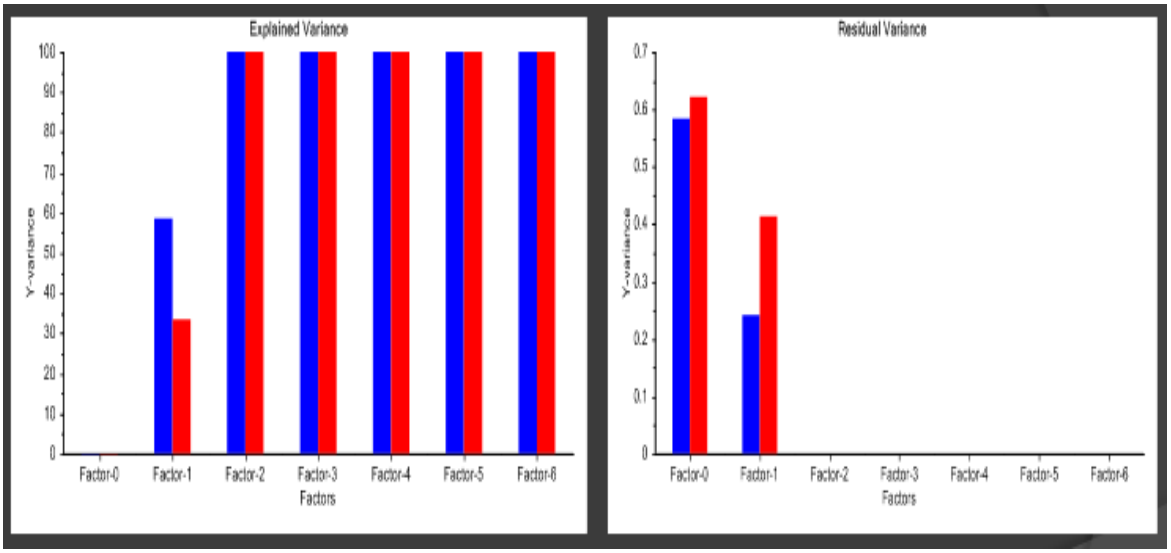

Figure 12.

Validation of Models

A) Predicted vs. Actual concentration plots for Ari

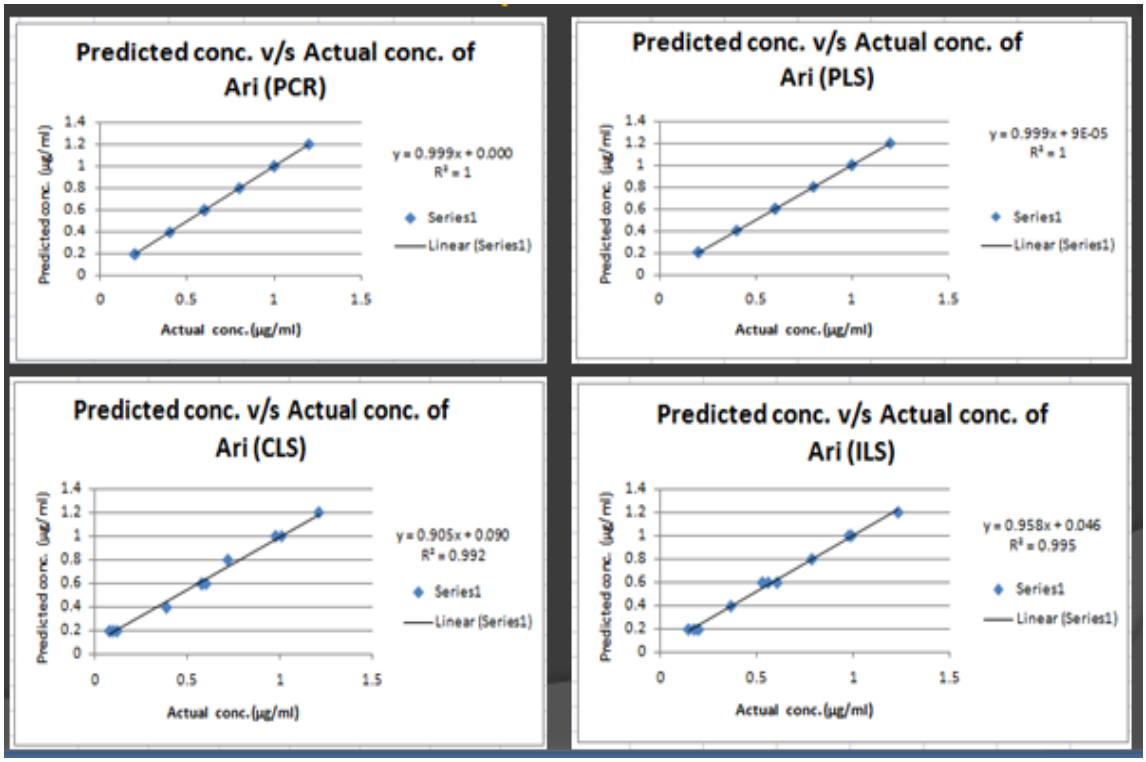

Figure 13.

Predicted vs. Actual concentration plots for Clz.

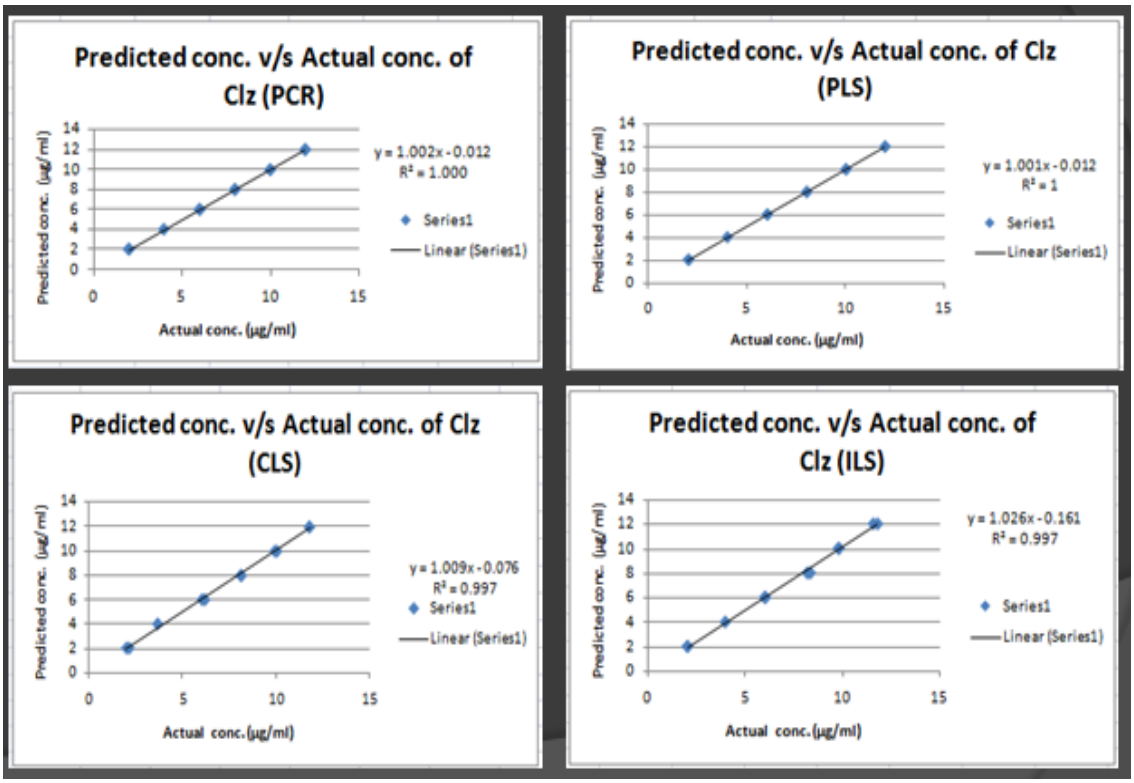

Figure 14.

B) Residual vs. Actual concentration plots for Ari.

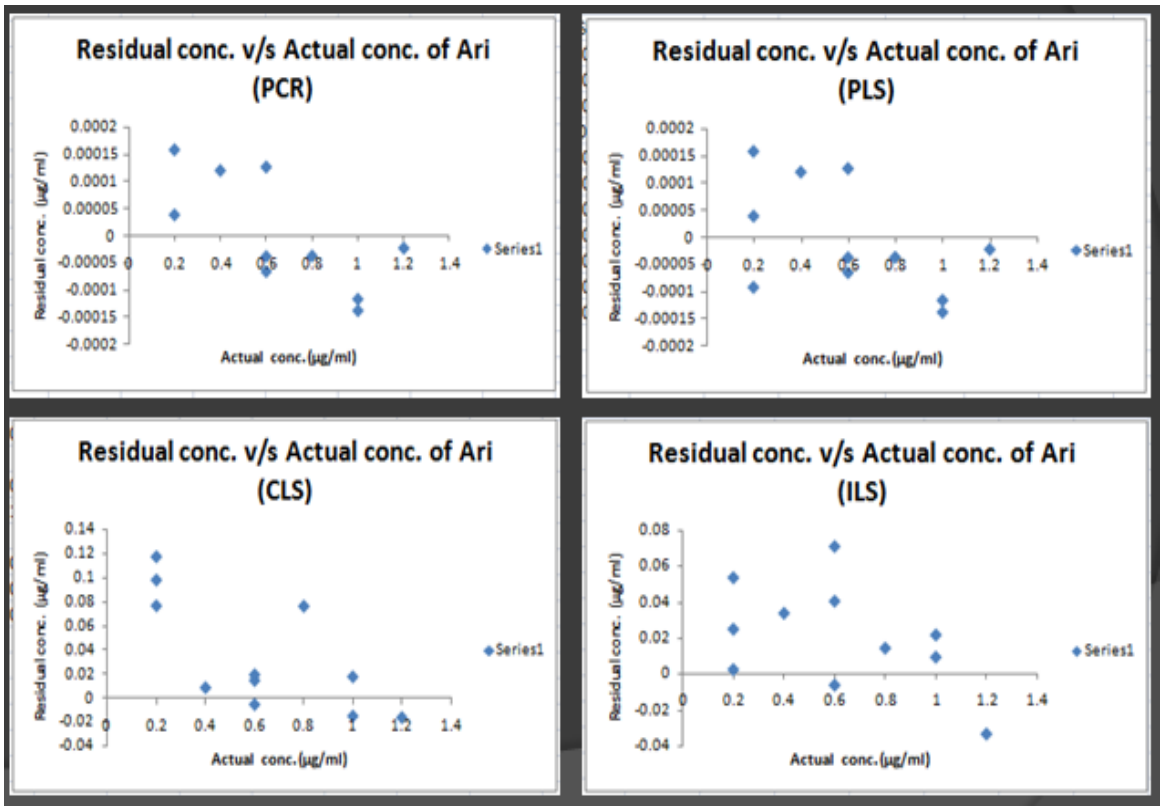

Figure 15.

Residual vs. Actual concentration plots for Clz.

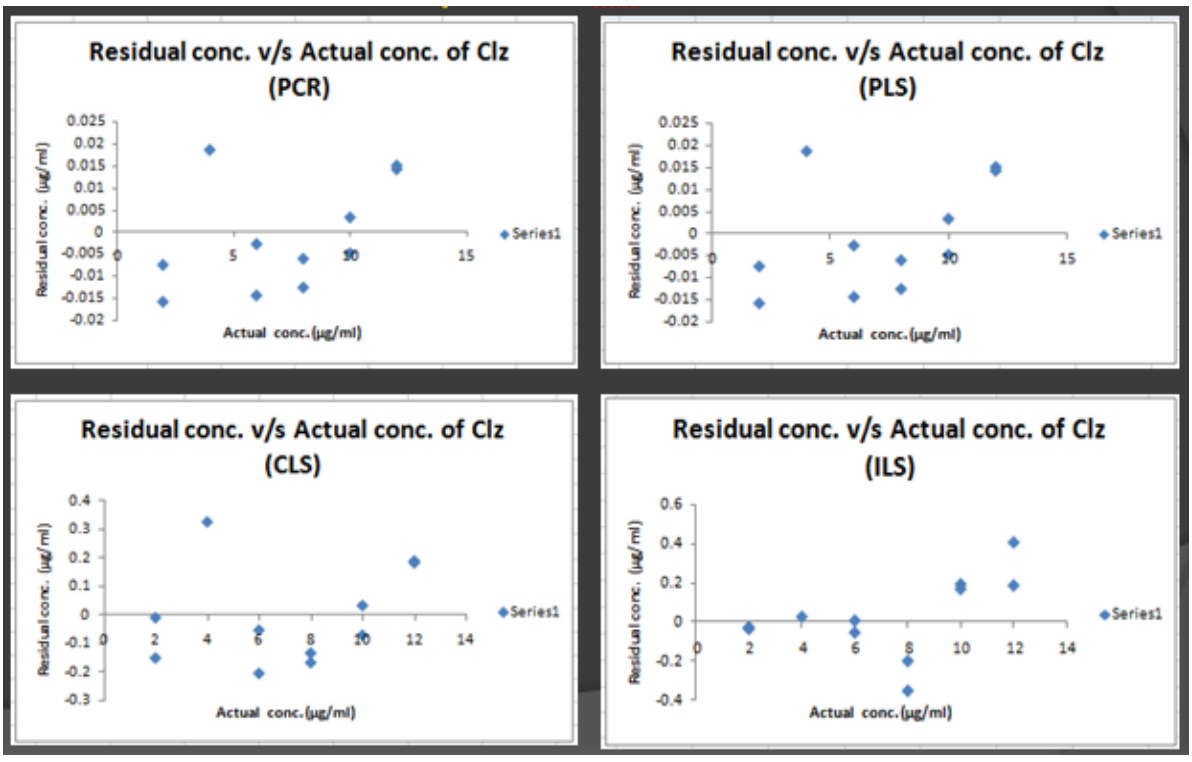

Figure 16

C) Root Mean Square Error of Prediction (RMSEP)value

- The predictive ability of the model can be defined as RMSEP. RMSEP summarizes both Precision and Accuracy (Table 20). It is used for examining the errors in the predicted concentration. It is calculated from following formula:

$$RMSEP = \sqrt{\frac{\sum_{I=1}^{N}(C_{Actual} - C_{Predicted})^2}{N}}$$

Where, N is the number of samples used for validation i.e. 11. The results of future predictions can then be presented as "predicted values ±2•RMSEP".

Table 20: RMSEP values

Drug	Methods			
	PCR	PLS	CLS	ILS
Ari	0.0009823	0.0009823	0.05766	0.03462
Clz	0.0007559	0.0007559	0.16397	0.19804

D) Applicability of the developed chemometric methods

- All four methods were successfully applied for the estimation of Ari and Clz in synthetic mixture (Table 21).

Table 21: Assay values

Methods	% Assay(mean)±SD, n=6	
	Ari	Clz
PCR	100.02±0.0031	100.19±0.0309
PLS	100.02±0.0031	100.19±0.0309
CLS	99.96±0.269	100.01±0.269
ILS	99.84±1.390	99.45±1.390

3) Development and Validation of both drugs in combination by QbD approach for isocratic RP-HPLC method

Screening design summary.

Design Summary

Study Type	Factorial	Runs	16
Initial Design	2 Level Factorial	Blocks	No Blocks
Center Points	0		
Design Model	2FI		

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	pH		Numeric	2.70	4.30	-1.000	1.000	3.500	0.800
B	Buffer type		Categoric	Phosphate	Acetate			Levels:	2
C	Aqueous ratio	ml	Numeric	42.00	52.00	-1.000	1.000	47.000	5.000
D	Flow rate	ml/min	Numeric	0.80	1.20	-1.000	1.000	1.000	0.200
E	Injection volume	ul	Numeric	10.00	20.00	-1.000	1.000	15.000	5.000

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Retention time 1	min	16	Factorial	2.370	16.927	6.661	3.906	7.142	None	RMain effects
Y2	Retention time 2	min	16	Factorial	2.563	35.323	11.461	9.560	13.782	None	R2FI
Y3	Asymmetry 1		16	Factorial	1.000	2.976	1.408	0.431	2.976	None	R2FI
Y4	Asymmetry 2		16	Factorial	1.091	2.382	1.371	0.374	2.183	None	R2FI
Y5	Theoritical plate		16	Factorial	2046.000	8725.000	5947.938	1644.683	4.264	None	R2FI
Y6	Theoritical plate		16	Factorial	2269.000	11730.000	6808.438	2622.641	5.170	None	R2FI
Y7	Resolution 1		16	Factorial	0.713	18.999	6.331	6.396	26.647	None	R2FI
Y8	Resolution 2		16	Factorial	0.758	17.207	7.639	5.867	22.701	None	R2FI

Figure 17.

Screening

Table 22: Screening values.

RUN	Factor 1 A : pH	Factor 2 B:Buffer type	Factor 3 C: % aqueous	Factor 4 D: Flow rate (ml/min)	Factor 5 E: Injection volume (µl)	Response 1: Retention time (Clz)	Response 2: Retention time (Ari)	Response 3: Tailing factor (Clz)	Response 4: Tailing factor (Ari)	Response 5: Theoretical plates (Clz)	Response 6: Theoretical plates (Ari)	Response 7: Resolution (Clz)	Response 8: Resolution (Ari)
1	2.7	Phosphate	42	1.2	10	4.387	6.023	1.243	1.148	4956	5377	11.43	5.68
2	4.3	Acetate	52	0.8	10	7.143	11.197	1.465	1.175	8725	11730	14.452	11.298
3	2.7	Acetate	52	1.2	10	2.813	3.627	2.976	1.091	3414	2425	0.951	0.758
4	2.7	Acetate	52	0.8	20	4.127	5.337	1.494	1.561	5582	4694	1.675	1.116
5	4.3	Phosphate	42	1.2	20	5.63	8.007	1.283	1.227	5040	5391	3.479	6.326
6	2.7	Phosphate	52	1.2	20	7.023	14.79	1.164	1.2	6005	7328	18.999	14.782
7	4.3	Acetate	42	0.8	20	5.32	6.807	1.529	1.244	7289	9621	2.327	5.659
8	4.3	Phosphate	42	0.8	10	8.29	11.797	1.283	1.282	6797	7079	2.089	7.302
9	4.3	Phosphate	52	1.2	10	13.877	31.91	1.307	1.313	6667	8059	18.942	17.207
10	4.3	Acetate	42	1.2	10	3.58	4.577	1.395	1.111	4931	6866	0.713	0.784
11	4.3	Acetate	52	1.2	20	4.77	7.49	1.5	1.234	6431	8919	4.184	9.825
12	2.7	Acetate	42	0.8	10	3.533	3.823	1.06	2.382	5385	3765	1.294	1.316
13	2.7	Phosphate	42	0.8	20	6.513	8.93	1.292	1.242	6288	6535	12.861	6.29
14	2.7	Phosphate	52	0.8	10	10.27	21.18	1.188	1.215	8217	9310	3.679	16.435
15	4.3	Phosphate	52	0.8	20	16.927	35.323	1.351	1.243	7394	9567	3.443	16.529
16	2.7	Acetate	42	1.2	20	2.37	2.563	1	2.261	2046	2269	0.78	0.913

Pareto charts for Retention time

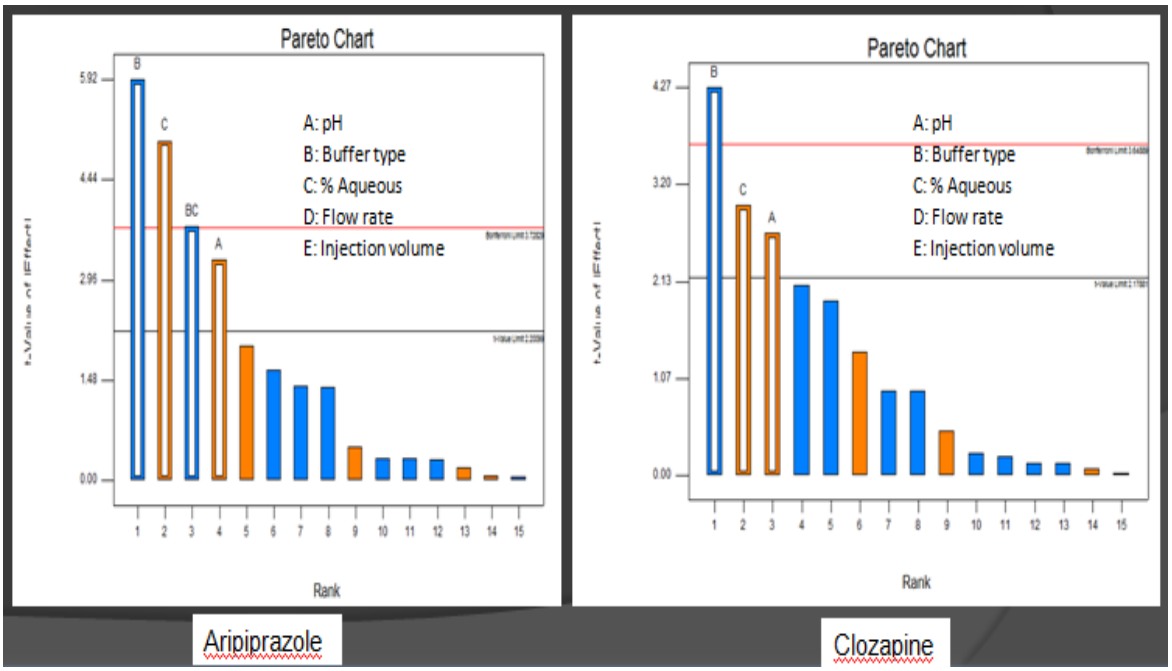

Figure 18.

Pareto charts for Theoretical plates

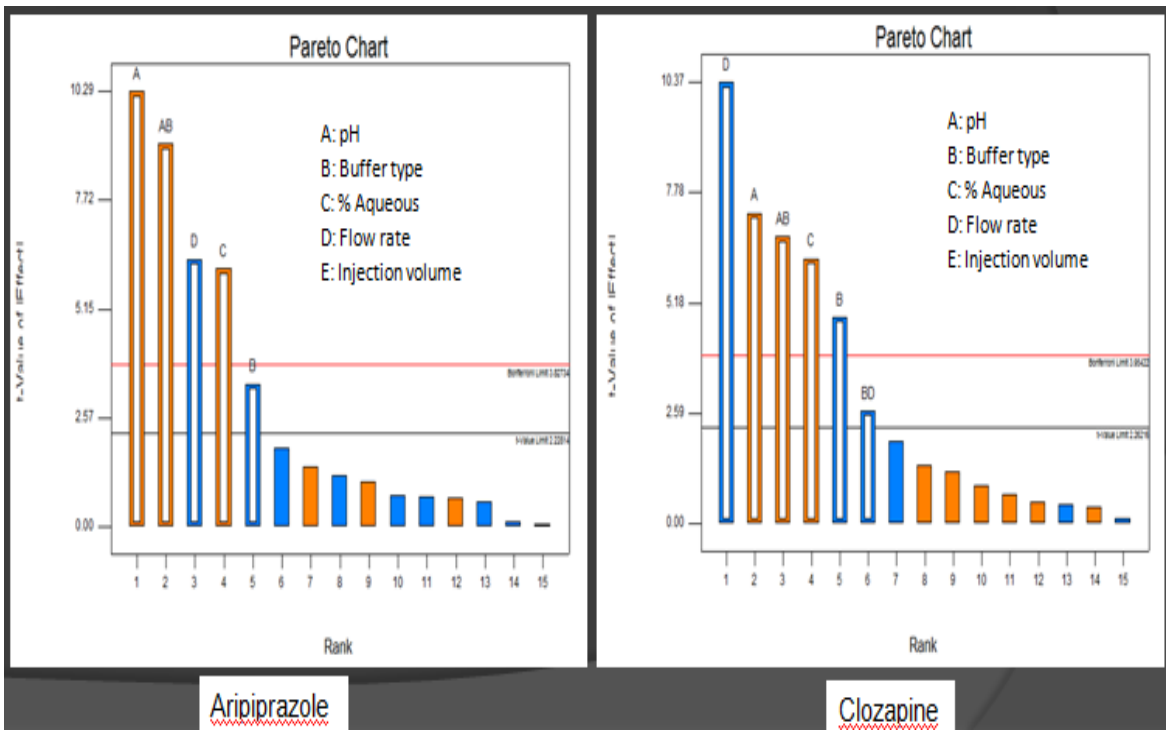

Figure 19

Pareto charts for Tailing Factor

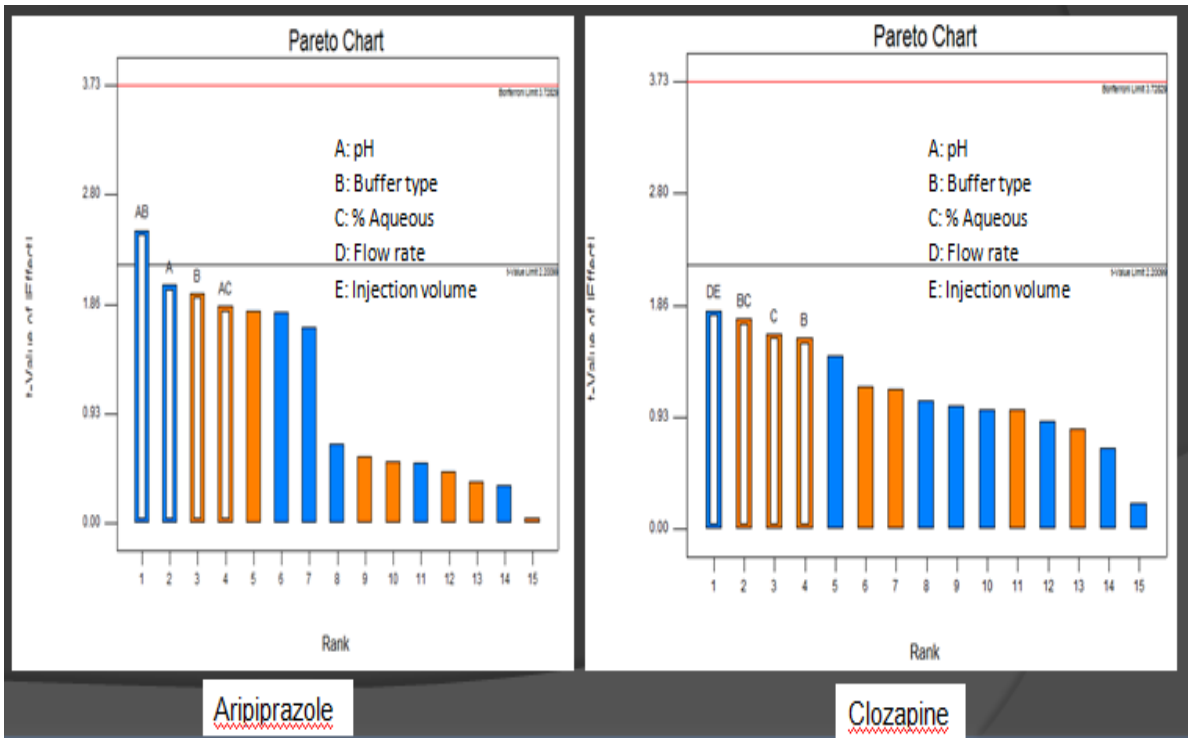

Figure 20

Pareto charts for Resolution

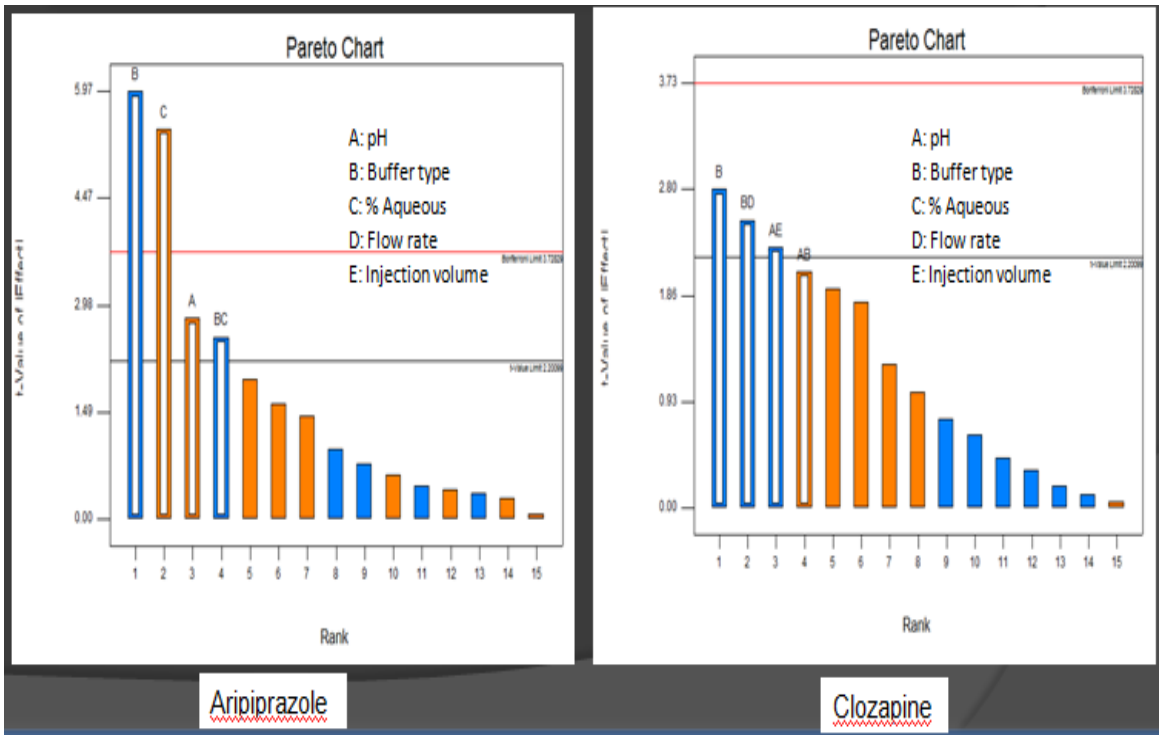

Figure 21

Optimization Design summary

Design Summary

Study Type	Response Surface	Runs	34
Initial Design	Box-Behnken	Blocks	No Blocks
Design Model	Quadratic		

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	pH		Numeric	2.70	4.30	-1.000	1.000	3.500	0.549
B	Aquous ratio		Numeric	42.00	52.00	-1.000	1.000	47.000	3.430
C	Flow rate		Numeric	0.80	1.20	-1.000	1.000	1.000	0.137
D	Buffer type		Categoric	Acetate	Phosphate			Levels:	2

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	RT 1		34	Polynomial	2.51	20.46	6.89	4.22	8.15	None	Quadratic
Y2	RT 2		34	Polynomial	2.89	59.03	11.80	11.39	20.42	None	2FI
Y3	AS 1		34	Polynomial	0.78	3.44	1.41	0.39	4.43	None	2FI
Y4	AS 2		34	Polynomial	0.88	3.18	1.28	0.35	3.63	None	Linear
Y5	TP 1		34	Polynomial	2644.00	9250.00	6256.94	1372.44	3.50	None	Quadratic
Y6	TP 2		34	Polynomial	2477.00	11073.00	7273.50	2043.98	4.47	None	Quadratic
Y7	RES 1		34	Polynomial	0.97	24.22	9.73	7.39	24.91	None	Quadratic
Y8	RES 2		34	Polynomial	0.78	21.10	7.83	4.58	27.02	None	Quadratic

Figure 22.

Optimization

Table 23: Optimization values

RUN	Factor 1 A : pH	Factor 2 B: % aqueous	Factor 3 C: Flow rate (ml/min)	Factor 4 D: Buffer type	Response 1: Retention time (Clz)	Response 2: Retention time (Ari)	Response 3: Tailing factor (Clz)	Response 4: Tailing factor (Ari)	Response 5: Theoretical plates (Clz)	Response 6: Theoretical plates (Ari)	Response 7: Resolution (Clz)	Response 8: Resolution (Ari)
1	2.7	42	1	Phosphate	5.307	7.37	1.238	1.172	5616	5857	11.8	6.19
2	3.5	42	1.2	Acetate	2.837	3.267	1.476	1.28	5504	6327	2.286	2.718
3	2.7	47	0.8	Acetate	3.733	4.293	1.155	1.408	4343	4343	1.47	2.305
4	3.5	47	1	Acetate	3.767	4.61	1.37	1.25	6908	7338	3.689	4.265
5	4.3	52	1	Acetate	5.57	8.377	1.514	1.224	7639	10400	5.636	9.646
6	2.7	42	1	Acetate	2.84	3.02	1	3.182	2644	3712	1.31	0.861
7	3.5	47	1	Phosphate	8.703	13.96	1.238	1.204	6714	7610	16.99	9.898
8	2.7	47	1.2	Acetate	2.51	2.89	0.775	1.531	3269	2477	1.967	0.781
9	4.3	42	1	Phosphate	7.003	11.327	1.564	1.275	4466	3562	13.404	7.358
10	4.3	47	0.8	Acetate	6.15	8.643	1.568	1.2	8185	11073	7.431	8.327
11	3.5	52	0.8	Acetate	5.41	7.38	1.469	1.233	8273	10441	6.739	7.499
12	3.5	47	1	Acetate	3.733	4.57	1.444	1.229	6381	7211	3.569	4.172
13	2.7	52	1	Phosphate	7.837	15.193	1.228	1.184	6821	8128	1.231	14.001
14	3.5	47	1	Phosphate	8.607	13.793	1.274	1.216	6745	7562	17.062	9.871
15	2.7	47	1.2	Phosphate	5.407	8.707	1.233	1.164	5390	6213	14.221	8.986
16	4.3	47	1.2	Phosphate	15.1	29.113	1.594	0.877	5261	8422	23.377	13.371
17	2.7	52	1	Acetate	3.287	4.077	3.436	1.458	4397	3186	0.972	3.252
18	4.3	47	0.8	Phosphate	15.727	35.043	1.624	0.885	5481	6290	24.135	14.801
19	3.5	47	1	Phosphate	8.34	12.675	1.25	1.188	7726	8316	17.326	9.261
20	2.7	47	0.8	Phosphate	7.873	12.613	1.255	1.2	7095	8094	12.716	10.169
21	3.5	47	1	Acetate	3.717	4.55	1.462	1.25	6726	7148	3.63	4.214
22	3.5	47	1	Acetate	3.697	4.527	1.423	1.25	6654	7463	3.615	4.258
23	3.5	47	1	Phosphate	7.753	11.773	1.268	1.202	6677	7196	15.764	8.625
24	3.5	42	0.8	Phosphate	8.427	11.947	1.35	1.224	6465	7261	11.174	7.203
25	3.5	52	1.2	Acetate	3.64	4.97	1.4	1.212	6066	7697	5.372	6.45
26	3.5	47	1	Acetate	3.697	4.527	1.462	1.29	6654	7463	3.615	4.258
27	3.5	42	0.8	Acetate	4.257	4.9	1.429	1.273	7815	8290	3.059	3.163
28	4.3	47	1.2	Acetate	4.1	5.767	1.5	1.189	6467	8564	5.21	7.375
29	3.5	52	1.2	Phosphate	10.83	21.343	1.253	1.243	7220	8445	21.69	14.652
30	4.3	52	1	Phosphate	20.46	59.027	1.369	1.009	5546	8465	17.851	21.101
31	4.3	42	1	Acetate	4.45	5.8	1.586	1.222	6491	9071	2.371	5.828
32	3.5	47	1	Phosphate	7.663	11.597	1.25	1.205	6523	7126	15.641	8.49
33	3.5	42	1.2	Phosphate	5.89	8.357	1.255	1.194	5324	5723	10.227	6.468
34	3.5	52	0.8	Phosphate	15.8	31.09	1.337	1.294	9250	10825	24.215	16.552

Contour 3D graphs for Retention time

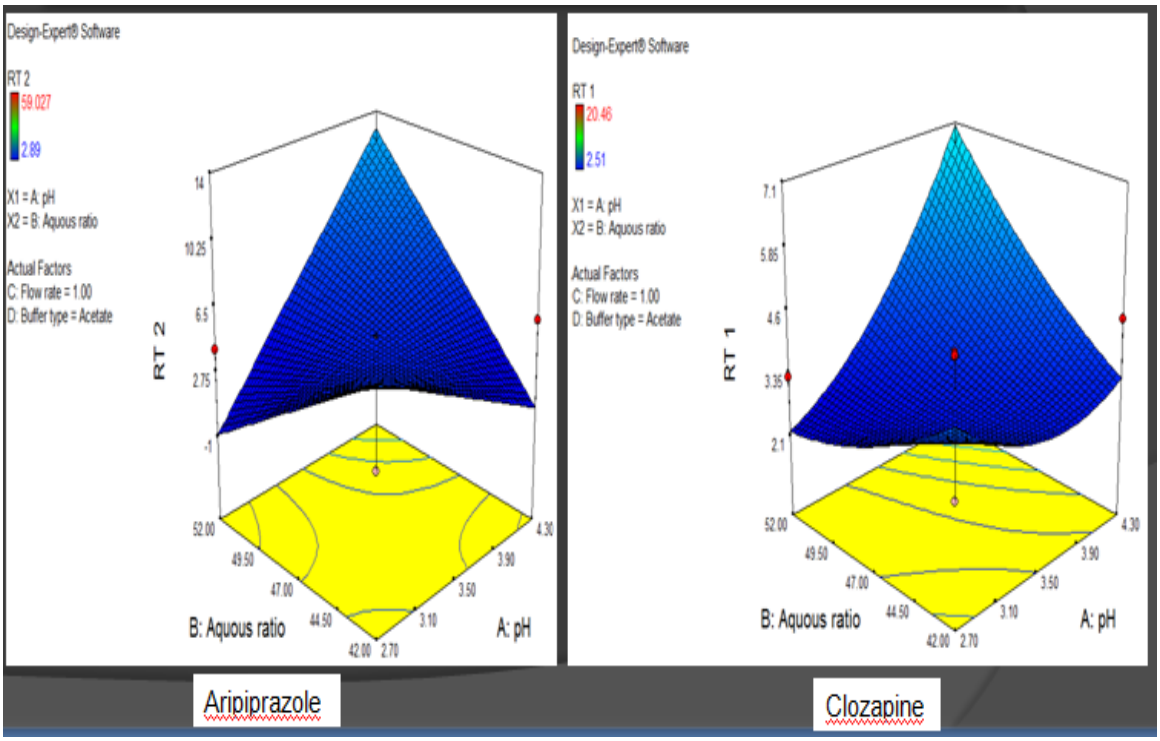

Figure 23.

Contour 3D graphs for Theoretical plates

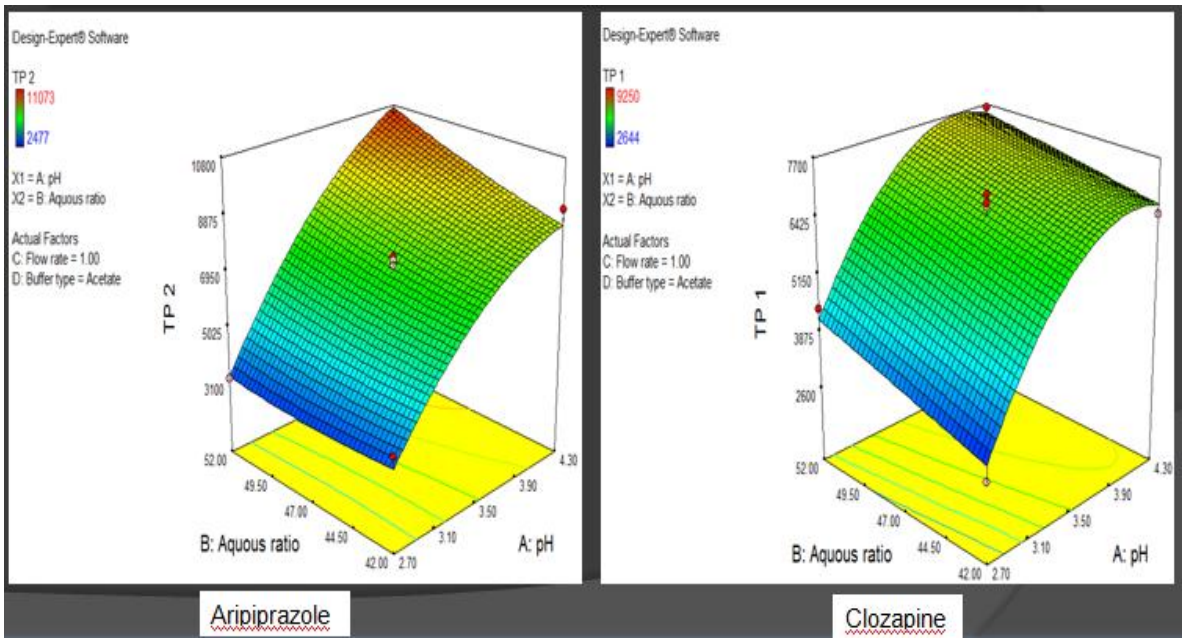

Figure 24

Contour 3D graphs for Tailing Factor (Assymetry)

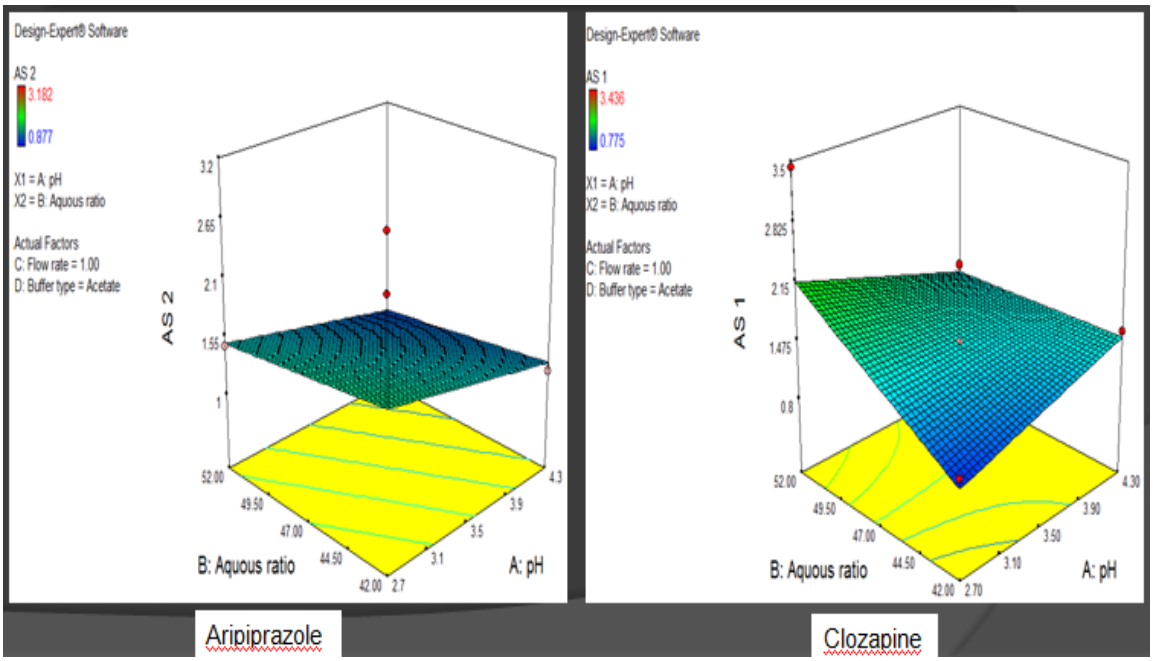

Figure 25

Contour 3D graphs for Resolution

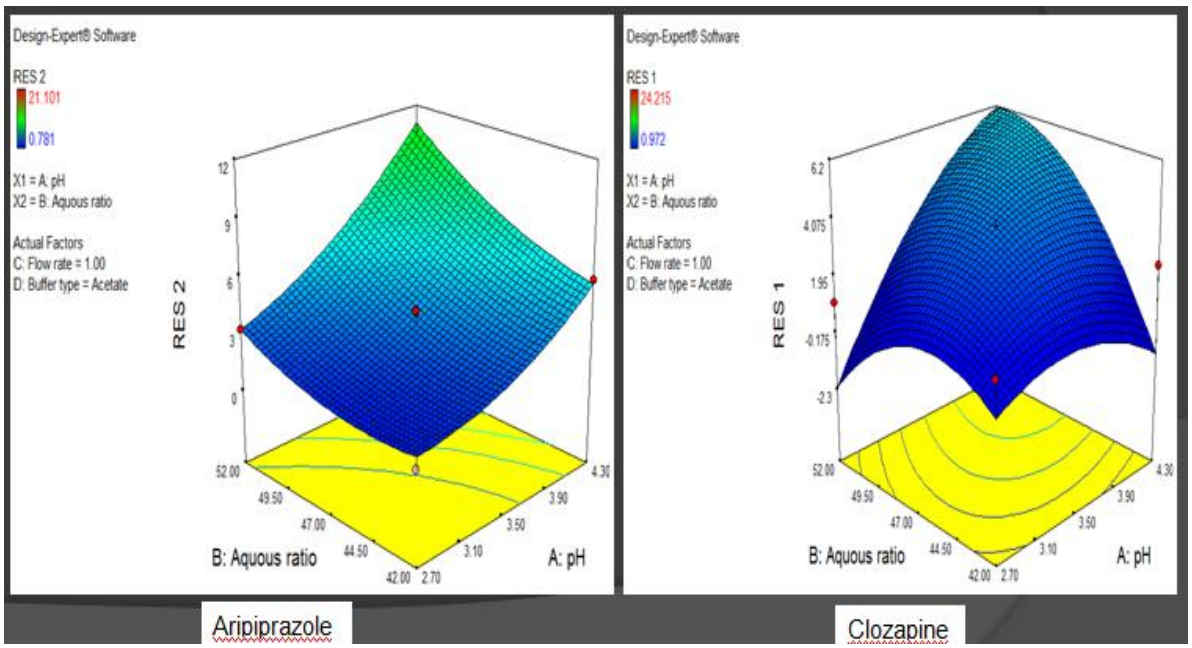

Figure 26

Desirability bars

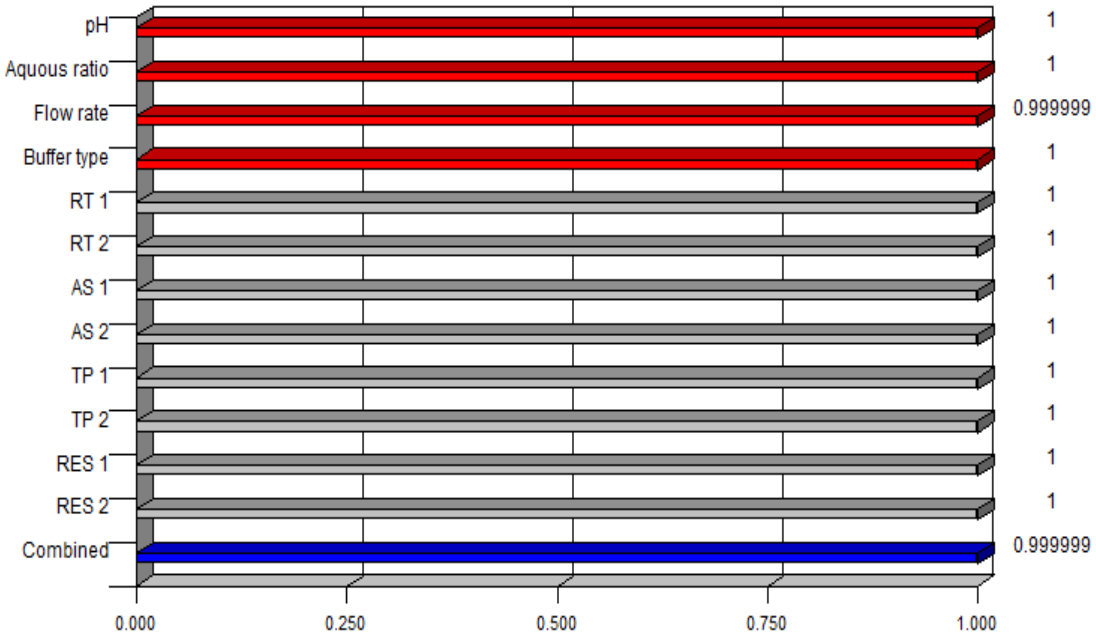

Figure 27.

Desirability contour

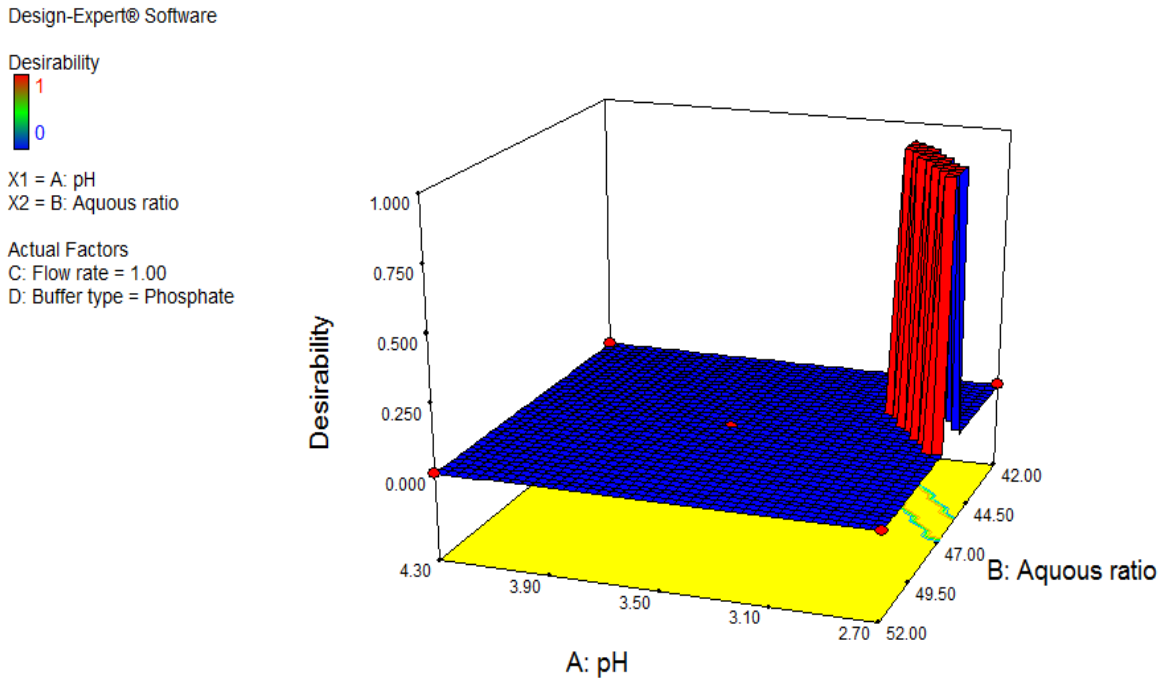

Figure 28.

Design space

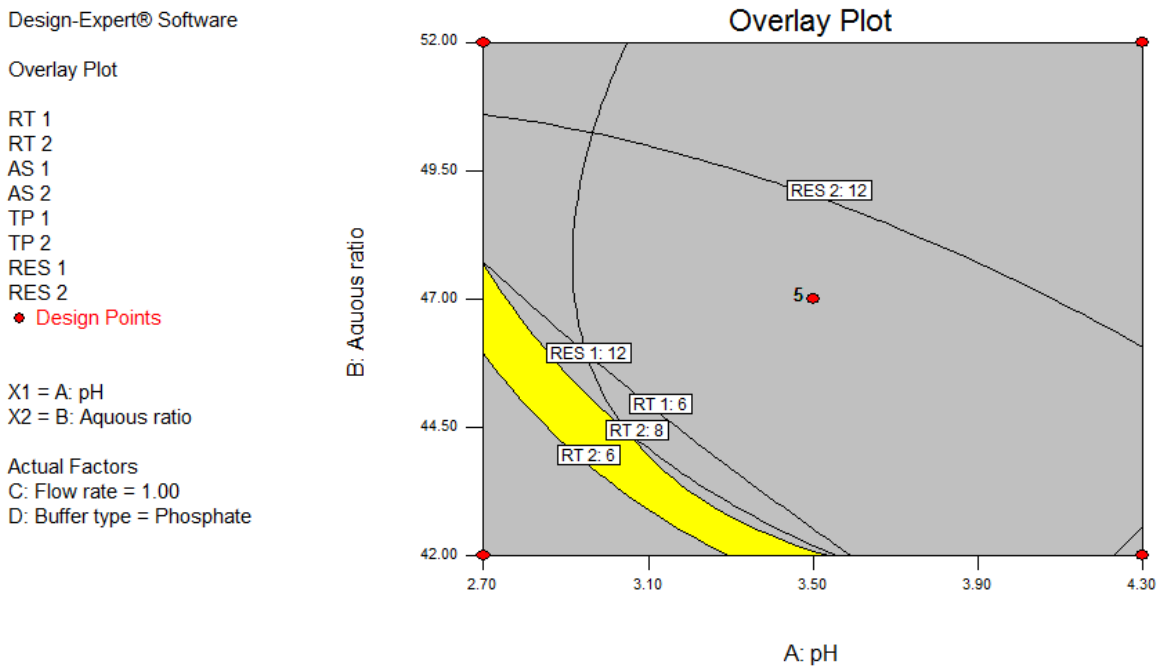

Figure 29.

Optimized Method Parameters

Table 24	
METHOD PARAMETER	OPTIMIZED VALUE
COLUMN	WATERS-C18 column, (Column dimensions: 250 mm x 4.6 mm,5 µm)
MOBILE PHASE	METHANOL: PHOSPHATE BUFFER (Potassium Dihydrogen Phosphate, pH- 2.93) = 55 : 45
FLOW RATE	1ml/min
RETENTION TIME	Ari – 9.347 min & Clz – 6.363 min
DETECTION WAVELENGTH	225 nm
TEMPERATURE	Ambient

Method Validation

1 Linearity

The calibration curve was constructed by plotting concentrations of Ari and Clz versus their respective peak areas, and the regression equations were calculated. The linearity of the method was investigated by using concentrations in the range 2.5 – 17.5 µg/ml for Ari and 25 – 175 µg/ml for Clz (Figure 30). Retention times for Ari and Clz were found to be 9.347 min and 6.363 min respectively (Figure 31).

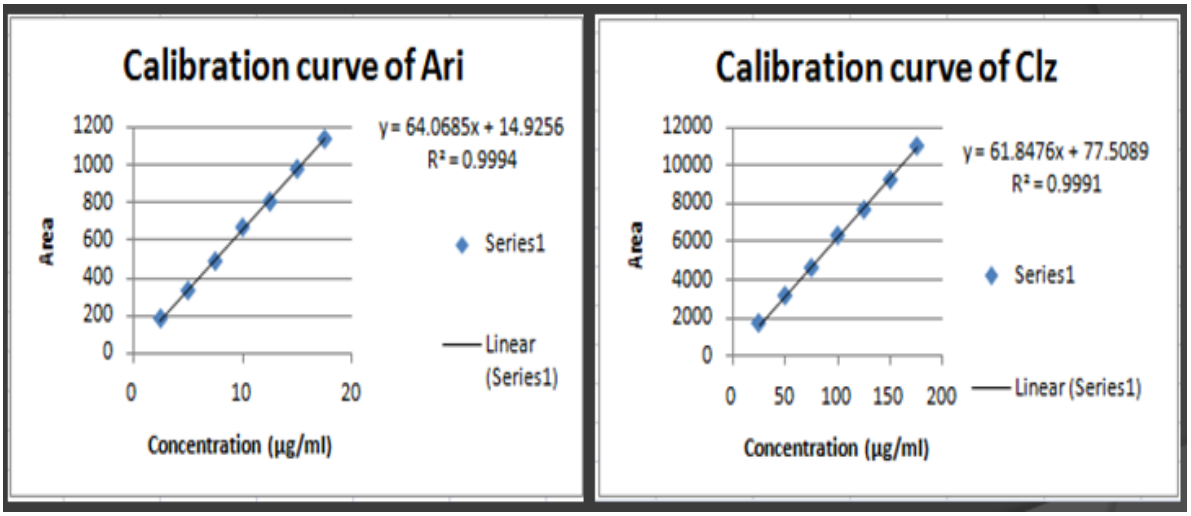

Figure 30: Calibration curve.

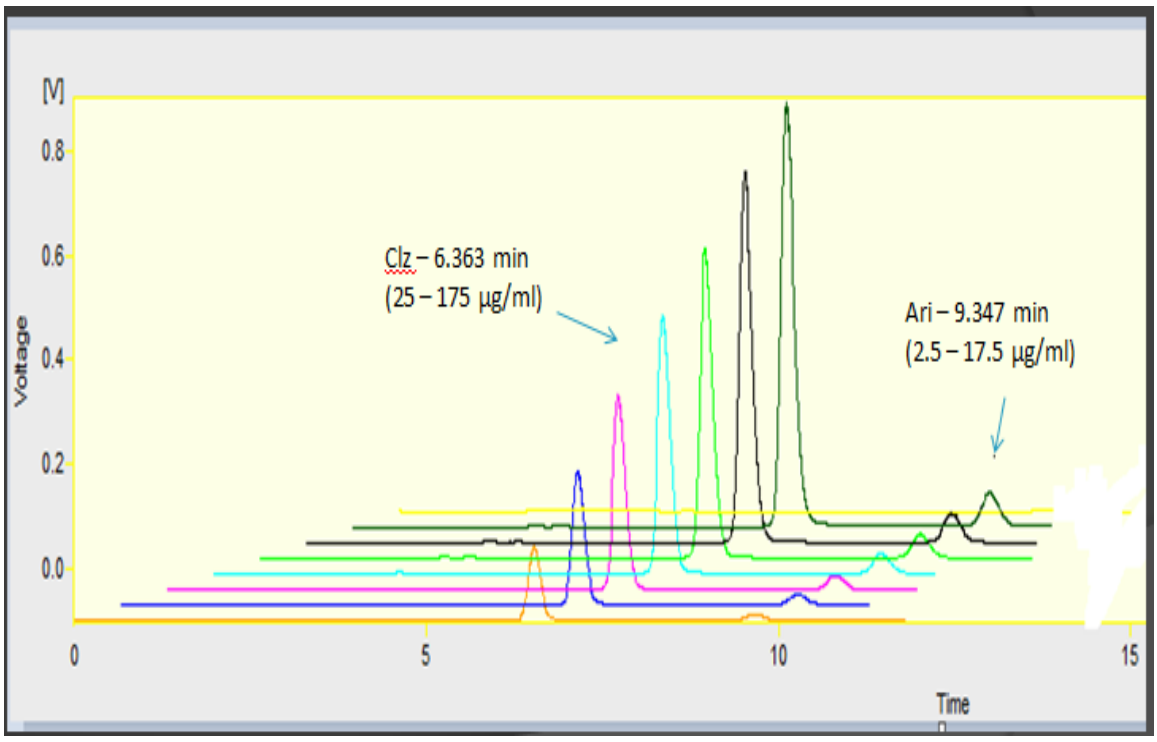

Figure 31: Calibration curve (3D chromatogram).

2 Accuracy (Recovery)

Accuracy of the method was studied using standard addition method at three different levels (80, 100, and 120%) by recovery experiments. Known amounts of standard solutions containing Ari and Clz were added to a prequantified sample solutions to reach 80%, 100% and 120% levels. Percentage Recovery was the mean of three determinations at each standard addition level (Table 25).

Table 25: Accuracy results								
% spiking	Concentration actual(µg/ml)		Concentration added (µg/ml)		Average concentration recovered (µg/ml)		%Recovery ± SD; n=3	
	Ari	Clz	Ari	Clz	Ari	Clz	Ari	Clz
80	5	50	4	40	4.065	41.724	101.635 ± 1.629	104.309 ± 1.744
100			5	50	4.931	51.162	98.612 ± 1.613	102.323 ± 1.147
120			6	60	5.874	59.961	97.904 ± 0.903	99.935 ± 0.394

3 Precision000000

To demonstrate agreement among results, a series of measurements were done. Three replicate injections of specific standard at various time intervals on the same day were injected into system for intraday precision and were repeated on three different days for interday precision. The % RSD (Relative Standard Deviation) of the results was calculated (Table 26).

Table 26: Precision results									
CONC. (µg/ml)		Inter-day precision				Intra-day precision			
		MEAN AREA ± SD; n=3		%RSD		MEAN AREA ± SD; n=3		%RSD	
Ari	Clz	Ari	Clz	Ari	Clz	Ari	Clz	Ari	Clz
2.5	25	182.691 ± 2.718	1741.38 ± 30.249	1.488	1.737	179.46 ± 2.31	1765.18 ± 18.423	1.287	1.044
10.0	100	661.013 ± 12.203	6296.8 ± 81.049	1.846	1.287	668.115 ± 2.477	6352.22 ± 20.791	0.371	0.327
17.5	175	1159.68 ± 22.819	11013.1 ± 175.656	1.968	1.595	1177.6 ± 14.074	11035 ± 149.836	1.195	1.358

4 Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) which determines the sensitivity of method are calculated by equation 1 and 2 (Table 27).

LOD =3.3 σ/S …….. (1)

LOQ =10 σ/S ……..... (2)

Where ‘σ ‘is the standard deviation of intercepts and ‘S’ is the slope of response.

Table 27: LOD and LOQ		
PARAMETER	Ari	Clz
LOD(µg/ml)	0.3178	2.5842
LOQ(µg/ml)	0.9631	7.8308

5 Robustness

- The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.
- If measurements are susceptible to variation in analytical conditions, the analytical condition should be suitably controlled or a precautionary statement should be included in the procedure.
- Robustness of the method was demonstrated by deliberately changing the chromatographic conditions like pH, mobile phase ratio and flow rate (Table 28).

Table 28: Robustness results

FACTOR		PEAK AREA(mV.sec)	
		Ari	Clz
A. pH	2.73	330.915	3154.581
	2.93	338.513	3187.917
	3.13	334.214	3215.000
	MEAN± SD	334.547 ± 3.810	3185.833 ± 30.263
	% RSD	1.139	0.950
B. Wavelength	0.9ml/min	332.717	3193.978
	1ml/min	336.077	3209.680
	1.1ml/min	337.427	3168.047
	MEAN± SD	335.407 ± 2.425	3190.568 ± 21.025
	% RSD	0.723	0.659
C. AQUEOUS RATIO	43 %	333.490	3211.038
	45 %	336.178	3189.876
	47 %	331.740	3158.751
	MEAN± SD	333.803 ± 2.235	3186.555 ± 26.301
	% RSD	0.670	0.825

6 System Suitability Parameters

System suitability testing was carried out on freshly prepared standard solution (n=6) of Ari and Clz. System suitability parameters obtained with 20µl injection volumes are summarized in Table 29.

Table 29: System Suitability results

PARAMETER	DATA OBTAINED	
	Ari	Clz
RETENTION TIME ± SD	9.423 ± 0.073	6.332 ± 0.053
Theoretical plate ± SD	5910.333 ± 132.672	5568.833 ± 141.623
Tailing factor ± SD	1.164 ± 0.020	1.264 ± 0.031
Resolution ± SD	6.538 ± 0.259	12.695 ± 0.147

7 Assay

Tablets were crushed to powder and it was dissolved in 10ml of methanol. Solution was centrifuged and filtered through 0.2um syringe filter. Aliquot of 1ml was drawn and volume was made up to 10ml with mobile phase and sample was injected into HPLC (Table 30).

Table 30: Assay results

Sample	Label claim	Avg Amount found	%Assay (Avg±SD); n=6	%RSD
Ari	10 mg	9.671mg	96.711 ± 0.364	0.377
Clz	100 mg	97.750 mg	97.750 ± 0.412	0.422

SUMMARY OF VALIDATION RESULTS FOR THE DEVELOPED METHOD.

Table 31: Summary

PARAMETER	Ari	Clz
Analytical wavelength (nm)	225 nm	
Retention time (min)	9.347 min	6.363 min
Beer's range (µg/ml)	2.5 – 17.5 µg/ml	25 – 175 µg/ml
Regression equation	y = 64.0685x + 14.9256	y = 61.8476x + 77.5089
Intraday precision (%RSD)	0.951	0,910
Inter day precision (%RSD)	1.767	1.540
LOD	0.3178	2.5842
LOQ	0.9631	7.8305
ACCURACY (%RECOVERY)	98.612	102.323
Assay	96.711 ± 0.364	97.750 ± 0.412

CONCLUSION

The results of present study indicate that the proposed UV spectroscopic methods and RP-HPLC method are simple, precise and accurate. Statistical analysis proves that the methods are repeatable and selective for the analysis of Aripiprazole and Clozapine in combination. It can therefore be concluded that the developed analytical methods can be use for routine Analysis of both the drug in combination.

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