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CHEMOMETRICS ASSISTED AND RP-HPLC METHODS FOR QUANTIFICATION OF NETUPITANT AND PALONOSETRON HYDROCHLORIDE BY QBD APPROCH: DEVELOPMENT AND VALIDATION.

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ABSTRACT

A simple, sensitive, accurate, rapid, precise and economical Chemometrics assisted and RP-HPLC method By QbD approach for simultaneous Quantification of Netupitant (NETU) and Palonosetron hydrochloride (PALO) have been developed and validated. Chemometrics offers a useful approach for quantification of combined dosage form. Analysis was performed at 225 to 280nm with interval of 6nm for Netupitant and Palonosetron hydrochloride respectively. Chemometrics method were found to be linear, (R^2=0.9998 & 0.9999) for all four methods CLS, ILS, PLS and PCR for NETU and PALO respectively. In HPLC method separation was achieved on Synchronies C-18 (250mm x 4.6mm, 5µm) column using mobile phase consisting mixture of 10 mM sodium acetate buffer, adjusted to pH 3.5 using Glacial acetic acid: acetonitrile: methanol in ratio of (10:50:40 v/v/v) pumped at flow rate 0.9 ml/min, at ambient temperature. QbD was applied for optimization of the method, Four variables buffer pH, wavelength, buffer ratio and flow rate have been varied and a model highlighting allowed design space was generated. By Applying DoE approach, a better chromatographic method was obtained and some of initially merged peaks in the chromatogram were resolved successfully and good symmetric peaks of NETU and PALO were also obtained. NETU and PALO were eluted at retention time of about 6.30 min, 3.55 min respectively. HPLC method was found to be linear

(R^2=0.9998) 6-36µg/ml for NETU and (R^2 = 0.9999) 01-0.06 µg/ml for PALO. Both methods Chemometrics and RP-HPLC by QbD showed acceptable results for simultaneous estimation of NETU and PALO.

KEYWORDS: Chemometrics assisted method, RP-HPLC, QbD approach, Netupitant, Palonosetron hydrochloride.

INTRODUCTION

D-AKYNZEO® is a formulation which contain Netupitant 300mg and Palonosetron (as Hydrochloride) 0.50mg, marketed as hard gelatin capsule in German market but not yet launched in Indian market.[1]

Netupitant(NETU) is a novel antiemetic agent used in cancer chemotherapy having IUPAC name-2-[3,5- bis(trifluoromethyl) phenyl]-N,2-dimethyl-N-[4-(2-methylphenyl)-6-(4-methylpiperazin-1-yl)pyridin-3-yl] propanamide with molecular weight: 578.61 g mol-1 and molecular formula is $C_{30}H_{32}$ F_6 N_{40} , pKa_1**:** 2.36, pKa_2**:** 7.65 and partition coefficient: 5.1. Structural formula is shown in figure-1.[1]

Palonosetron hydrochloride (PALO) is a antiemetic and antinauseant agent with IUPAC name -(3aS)-2-[(S)-1- Azabicyclo [2.2.2]oct-3-yl]-2,3,3a,4,5,6- hexahydro-1-oxo-1*H*benz[*de*] isoquinoline hydrochloride. Having molecular weight: 332.87, molecular formula: $C_{19}H_{24}N_{20}$.HCl, pKa**:** 8.9 and partition coefficient 4.3 at pH 7.4. Palonosetron hydrochloride exists as a single isomer and Its structural formula was mentioned in (figure-1).[2]

Netupitant is a selective high-affinity antagonist of human substance P/neurokinin 1 (NK1) receptors. Palonosetron is a selective serotonin subtype 3 (5-HT3) receptor antagonist with a strong binding affinity for this receptor. Cancer chemotherapy may be associated with a high incidence of nausea and vomiting, particularly when certain agents, such as cisplatin, are used. 5-HT3 receptors are located on the nerve terminals of the vagus in the periphery and centrally in the chemoreceptor trigger zone of the postrema area. It is thought that chemotherapeutic agents produce nausea and vomiting by releasing serotonin from the enterochromaffin cells of the small intestine and that the released serotonin then activates 5-HT3 receptors located on vagal afferents to initiate the vomiting reflex. The development of acute emesis is known to depend on 5-Hydroxytryptamine serotonin (5-HT) and the 5-HT3

receptor has been demonstrated to selectively participate in the emetic response. Delayed emesis has been largely associated with the activation of tachykinin family neurokinin 1 (NK1) receptors (broadly distributed in the central and peripheral nervous systems) by substance P. As shown in *in vitro* and *in vivo* studies, Netupitant and Palonosetron HCL can contribute to the inhibition of substance P mediated response.[3]

Literature survey suggests that methods have been reported for estimation of Palonosetron HCl by RP-HPLC[4], Stability indicating studies by HPTLC[5], some spectroscopic methods[6], liquid chromatography-tandem mass spectroscopy for estimation of Palonosetron in human plasma[7]. But for the Netupitant no analytical methods were reported.

Present research work is aimed to introduce a multivariate calibration of four Chemometric analytical methods and RP-HPLC by QbD approach for the quantification, development and validation (as per ICH guidelines)[8] of Netupitant and Palonosetron HCl.

EXPERIMENTAL

Instruments and Software

A Shimadzu model 1700 (Japan) double beam UV-Visible spectrophotometer with spectral width of 1 nm, wavelength accuracy of ± 0.1 nm and a pair of 10 mm matched quartz cell was used to measure absorbance of all the solutions. Spectra were automatically obtained by UV-Probe system software (Ver.2.1). The samples were weighed on electronic analytical balance (A×120, shimadzu). All the Chemometric calculation were done using the software MATLAB Version 6.1.0.450 Release 12.1, The Mathworks, Inc. (for CLS and ILS) and The unscrambler X 10.1, CAMO software (for PCR and PLS).

HPLC was performed on isocratic Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector Data acquisition, Rheodyne 7725 injector with fixed loop of 20 µl and integration was performed using Spinchrom software (Spinco biotech, Vadodara), separation was achieved on Synchronies C-18 (250mm x 4.6mm, 5µm). QbD was perform by Deign of expert® version 7.00 Microsoft excel and Microsoft office.

Reagents and Chemicals

Palonosetron hydrochloride (pure API) was purchased from Swapnroop pharmaceutical & Ltd. Aurangabad and Netupitant (pure API) was provided as a gift sample from Apicore

drugs pvt. Ltd. Baroda. and synthetic mixture as specified in German marketed formulation Akynzeo (300mg NETU and 0.500 mcg PALO) was made in pharmaceutical laboratory at MSU, methanol AR grade and HPLC grade (Rankem). Acetonitrile HPLC grade (Fischer). Double distilled water was filtered by 0.2µm membrane filter paper by vacuum filtration.

Selection of common solvent

After checking the solubility of drugs (NETU and PALO) in different solvents methanol has been selected as common solvent for developing spectral characteristics.

Selection of detection wavelength

Selection of analysis wavelength for NETU and PALO was done by scanning solutions from 200 to 400 nm. It shows that absorbance was maximum at 258nm for Netupitant and 241 nm for Palonosetron HCl. Detection wavelength selected for estimation of both drugs was 241 nm for RP-HPLC method**. (Figure.5)**

Preparation of standard stock solution

An accurately weighed 10mg of standard NETU and PALO powder were transferred in separate 100 ml volumetric flask and dissolved in methanol. After complete dissolution volume was made up to mark with methanol to give stock solution containing 100 µg/ml (final stock) of each NETU and PALO.

Preparation of synthetic mixture

Synthetic mixture was prepared by using Active ingredient (NETU 300 mg and PALO 0.50mg) and excipients include microcrystalline cellulose, sucrose laurate, povidone, croscarmellose sodium, silicon dioxide, sodium stearylfumarate, magnesium stearate. Glyceryl caprylate, polyglyceryl -3 dioleate, butylated hydroxyanisole. etc. Synthetic mixture was mixed properly, dissolved in 100 ml methanol, filtered and used for further analytical process.

(A) Chemometrics assisted methods for simultaneous quantification of Netupitant & Palonosetron hydrochloride[9,10,11,12]

Chemometric methods are multivariate analysis i.e. considers more than one variable at a time. Here, present paper consider absorbance at 10 different wavelengths (225 to 280 nm with interval of 6.0nm) 10 variables in contrast to other univariate methods described where absorbance at only one wavelength is considered (Absorbance matrix ‘A’).

Four different Chemometric methods

(1). Classical Least Squares (2). Inverse Least Squares (3). Principal Component Regression (4). Partial Least Squares or Projection to Latent Structure were studied.

Preparation of Calibration Set mixture

Total twenty-two binary mixture standards with required concentrations **(Table 1)** were prepared from stock solutions.

Preparation of Validation Set mixture

Total fourteen binary mixture standards with required concentrations **(Table 1)** were prepared from working solutions.

Producing Absorbance matrix 'A'

Absorbance matrix 'A' was produced by measuring the absorbance at 10 different wavelengths in uv spectrum region between 225 nm to 280 nm with wavelength interval of 6.0 nm. This region was selected because it contain most relevant information about both NETU and PALO drugs. The spectra of prepared binary mixture standards were recorded in the range of 225 nm to 280 nm with 6 nm interval**. (Figure 2).**

Principle Component Regression (PCR)

In PCR method dimensionality and complexity is reduced and is applied for spectrophotometric analysis, absorbance matrix concerns that a large pool of data contains 'n' variables (wavelengths, one column for each) and 'm' samples (calibration standards, one row for each). Each sample can be described as a point in 'n' dimensional space according to its absorbance values at different wavelengths. Thus, one sample is described by 'n' variables. PCR is a tool that reduces number of variables to only a few components, referred as a principal components (PCs). They are computed in such a way that the first PC is the one that carries most information (or in statistical terms: most explained variance). The second PC will then carry the maximum share of the residual information (i.e. not taken into account by the previous PC) and so on. All the PCs will be orthogonal to each other so, they are ranked in a manner so that first PC will explain maximum variance in the data. Theoretically, there can be as many PCs as the number of actual variables (n). But only first few PCs are considered because they explain almost all the variance in the data. After reducing the dimensionality of data to a few PCs, next step is regression to relate these PCs with

concentration. There will be individual regression for each drug present in the sample. Thus, PCR deals with only one response variable (concentration) at a time.

Partial Least Squares (PLS)

PLS model simultaneously determine both the X and Y matrices. X that can best predicts latent variables at Y. This model refers as projection to latent structures. This PLS latent variables model was similar to principle component and will be referred to as PCs or factors. In PCR we found PCs only for X variables and this PCs directly related to Y (sample concentration). In PLS were computed for X and Y both. PC of X was related to PC of Y. PLS can handle multiple variables in Y at a time in contrast to PCR. The difference between PCR and PLS both the method lies only in the Algorithm. PLS uses information lying in the both X and Y in order to fit the models. It adjust between X and Y iteratively to find the PCs. so PCs often requires optimal solution because it mainly focused on prediction of Y variables. (not on achieving the best projection of X as in PCR).

Classical Least Squares(CLS)

The CLS model assumes that Beer's law with the absorbance at each wavelength being proportional to the sample concentration. In matrices notation Beer's law model for 'm' calibration standards containing 1 chemical component with spectra of 'n' digitized absorbance was given by:

A=C*K

Where A= m x n matrix of calibration spectra.

C= m x n matrix of sample concentration.

K= 1 x n matrix of absorptivity, which represent the matrix of standard sample spectra at unit concentration and unit path length. so, the spectral matrix A was represented as product of smaller matrices C and K. The pure sample spectra (rows of K) are the factor loadings (also refer loading vectors) and chemical concentrations (columns in C) are the factors. In simplified way, it can be said that each component have specific absorptivity at each particular wavelength being considered. Each row of K matrix represents spectrum of one pure component at its unit concentration. K matrix will have as many rows as components and as many columns as wavelengths. Once we have matrices A and C, we can determine K by following equation:

K = pinv (C) * A

Where, pinv (C) = pseudo inverse of concentration matrix C.

For predicting unknown concentration of a mixture from its absorbance matrix, following equation is used:

C = A * pinv (K)

Inverse least square (ILS)

The ILS model assumes that concentration is a function of absorbance. The inverse of Beer's law model for m calibration standards with spectra of n digitized absorbance is given by.

C = A * P

P = n × 1 matrix of unknown calibration co-efficient relating the 1 component concentrations of the spectral intensities.

Since in ILS the number of wavelengths cannot exceed the total number of calibration mixtures, stepwise multiple linear regressions have been used for selection of wavelengths. Once we have matrices A and C, we can estimate P by following equation, where Pseudo inverse of matrix A is calculated,

P = *pinv* (A)*C

Pseudo inverse of matrix A is calculated instead of inverse as explained in Classical Least Squares method. (We can calculate P for as many components as we provide in matrix C i.e. if all the components in matrix C are not known, P will be calculated only for those components which are known and present in matrix C). For predicting the unknown concentration of a mixture from its absorbance matrix following equation is used:

C = A * P

Validation of ILS, CLS, PCR and PLS Models

Predicted vs. Actual Concentration Plot

The Predicted concentration of validation set was plotted and compared with the Actual concentration values. This tool is used to investigate whether the model accounts for concentration variation in the validation set or not. Plots were expected to fall on straight line with slope of 1 and 0 intercept. The predicted vs. actual concentration plots of prepared validation samples are shown in **Figure 3.** It was noticed that NETU and PALO in all samples lies on straight line & the equations of these lines are shown on the graph. This indicate that the prediction ability of the validation set is very much better in terms of recovery.

Residual vs. Actual concentration

The difference between the actual and residual concentration were plotted and compared against the actual concentration of validation set values. This tool is used to investigate whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict the future sample. For the validation set it can be found that the residual values more closer to zero & more randomly distributed. Concentration of residual vs. actual concentration plots for all methods is shown in **Figure 4.**

Applicability of sample

The selectivity of the proposed method was also assessed by the analysis of synthetic mixtures, the result of synthetic mixture study were mentioned in **Table.2,** where satisfactory results were obtained over the stated calibration range. In this experiment range of wavelength selected for analysis is 225–280 nm with the interval of 6 nm from the absorbance amount, the drug samples were computed and results were shown in **Table 2.**

Recovery

To examine the predictive ability of the calibration methods, the simultaneous estimation of the prediction set containing 12 samples of various concentrations of NETU and PALO were carried out by addition of NETU & PALO (300mg & 0.500mcg) in the blank matrix. The maximum values of the mean percent errors corresponding to CLS, ILS, PCR and PLS for the same matrix were completely acceptable because of their very smallest values. The mean recoveries and the relative standard deviations of four proposed methods were computed and indicated in **Table. 3-4.**

Their numerical values were completely acceptable because of their smallest values and hence found satisfaction for validity of all calibration methods. The linearity of the proposed Chemometric method for determination of NETU and PALO was evaluated by analyzing a series of different concentrations of standard drug. The linearity was found to be ranging between (6-36 µg/ml) for NETU and (0.01- 0.06 µg/ml) for PALO. Each concentration was repeated three times. The observed results were compared with expected results. The better mean recoveries and relative standard deviation mentioned in **(Table 3-4)** that suggested good accuracy of the proposed methods and no interference from formulations excipients

Statiscal Parameters[13,14,15]

Root Mean Square Error of Prediction (RMSEP): The predictive ability of regression model is described in different ways. The most general expression in the standard error of prediction (SEP) and standard error of calibration was denoted by RMSEP which summarizes both precision and accuracy. It is used for quantifying the errors in the predicted concentration. Calculated from following formula.

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

Where, N=the number of samples used for validation i.e. 14. The results of future predictions can then be presented as "predicted values ±2•RMSEP". RMSEP for NETU and PALO by Chemometric Methods are shown in **Table 5.**

B) RP-HPLC METHOD BY QbD APPROACH

Quality by Design (QbD) approach has been introduced by FDA for the pharmaceutical development to ensure a predefined product quality. Application of Quality by Design concept to the analytical method development leads to a more robust method. ICH guidelines Q8(R2) defines QbD as "A systematic approach to development that begins with predefined objectives and emphasizes product and process control, based on sound science and quality risk management". In this approach potential method variables that affects the overall quality of method are defined, their interactions are studied, control strategy is implemented and finally the method is continually monitored.[16-17]

Method development by QbD approach[18]

Various Sodium acetate buffer ratio (ml) with methanol and acetonitrile, pH of acetate buffer, wavelength and flow rate were tried to obtained well resolved, sharp peak, but, resolution and asymmetry of chromatogram was not acceptable. **(Figure 6).**

Hence, QbD approach was applied to get better resolution, reduced asymmetry and sharp chromatogram of the standard drugs and optimization of such robust method to get good results of Palonosetron HCl and Netupitant.

Method development using Quality by Design approach can be divided by following steps:

1). Definition of method goals 2). Risk assessment 3). Design of experiment with optimization 4). MODR (Method Operable Design Region), working point selection and

verification 5). Method Control Strategy based on the knowledge gained about the developed method.

Definition of method goals

Primary aim was to develop and validate a more robust method for simultaneous quantification of NETU and PALO Applying Quality by Design approach to get MODR (Method Operable Design Region). MODR is defined as the set of method parameters over which the robustness and ruggedness experimentation has shown that the method can meet requirements of ATP.

Risk assessment

In this stage of QbD approach, critical parameters that affect the overall quality of method were identified, such as pH of buffer solution, ratio of sodium acetate buffer, wavelength and flow rate for simultaneous quantification of NETU and PALO**. (Table 6)** .

Design of experiment

In design of experiment, four factors are considered: pH of buffer solution, ratio of buffer, wavelength and flow rate which were varied at four different levels. The critical responses studied were asymmetry, resolution, area and retention time of both drugs NETU and PALO. By employing various rational combinations of the identified critical factors, DoE generates some trials. From the data obtained by generated trials, DoE generates models for them and helps to simultaneously understand the influence of more than one factor on selected critical responses. For analysis of overall effect of all critical factors contour plots were generated that shows simultaneous effect of critical factors on selected response (**Figure 7**. Retention time of NETU and PALO, **Figure 8** Asymmetry of both drug, **Figure 9**. Resolution of NETU and PALO).

Optimized phase

Optimization criteria was selected for optimization of method and out of various solutions generated by software five solutions were selected for check point analysis.

Point verification and working point selection

Selected solutions were experimentally performed and final working point was selected, Predicted data and experimentally obtained data for selected solutions were tabulated in **(Table 7).** Desirability plot for selected working point is shown in **(Figure 10).**

Design space

Overlay plot **(Figure 11)** indicates design space region in which critical parameters can be avoided. Chromatogram obtained from selected working point has been shown in **(Figure 12).**

Final optimized chromatographic method

Column: Synchronies C18 (250 × 4.6 mm; 0.5 µm particle size)

Mobile phase: Sodium acetate buffer, pH 3.5(10 mM): Acetonitrile: Methanol (10:50:40% v/v/v), Flow rate: 0.9 ml/min, Temperature: Ambient, Detection wavelength: 241 nm.

METHOD VALIDATION

Developed method was validated according to ICH Q2 (R1) guidelines.

Linearity

From standard solution of NETU (6-36 µg/ml) and PALO (0.01-0.06 µg/ml) were prepared. Regression equation, Correlation coefficient, Slope and Intercept were calculated. Overlay chromatogram of NETU and PALO is shown in **(Figure 13).**

Precision

Three sample were prepared and analyzed as per the test method on same day and different days and calculated the %RSD. The average %RSD of intra-day and inter-day measurements for determination of both the drugs was found to be less than 2.

Accuracy

It was done by recovery study. Sample solution were prepared by spiking at about 80%, 100%, 120%.

LOD and LOQ

LOD and LOQ of developed methods were calculated from the equations given by ICH guidelines.

$LOD = 3.3*\sigma/S$

$LOQ = 10*\sigma/S$

where, σ= standard deviation of intercept, S=slope of calibration curve.50

Robustness

Robustness of method was determined in the form of Standard Deviation of retention time by small deliberate changes in flow rate, pH of buffer solution and detection wavelength.

System suitability parameter

System suitability parameters were studied to verify the optimum conditions. This is an integral part of liquid chromatographic method for assuring adequate performance of the system different parameters are evaluated such as resolution, capacity factor, separation factor, theoretical plates and asymmetry.

Applicability of developed method

Synthetic mixture was taken as Powder equivalent 300 mg NETU and 0.50 mg of PALO was weighed and transferred to 100 ml volumetric flask. 10 ml methanol was added and sonicated for 10 minutes. After sonication solution was diluted to 100 ml with sodium Acetate buffer pH 3.5 and filtered through Whatman filter paper grade 1. Appropriate dilution was made in the range of (6-36 µg/ml) of NETU, (0.01-0.06µg/ml) of PALO and solution was subjected to analysis.

RESULTS AND DISCUSSION

The calibration set of 24 standard binary mixture solutions which contain the concentration with the different ratio of NETU and PALO were randomized prepared in the linearity range of two mixture drugs. The uv absorbance data was obtain in the wavelength region of 225-280 nm with 6 nm interval. The Chemomatric calibrations were validated and calibrated in the CLS, ILS, PCR, PLS algorithms. The quality of multi-component analysis is dependent on the wavelength range, spectral mode used, calibration set chosen and calibration range.

(Figure 3) shows that the Zero-order overlay absorbance spectra of NETU and PALO and their standard binary mixture solution in methanol in the range of 200-400 nm region. This Zero order absorbance spectra of NETU and PALO totally overlapping to each other and making it complicate for use of conventional spectrophotometric techniques for simultaneous quantification, For this reason to solve overlapped spectra, four Chemometric methods have been applied.

RP-HPLC method was successfully validated as per ICH Q2(R1) guidelines. Beer's law was obeyed in range of, (6-36 µg/ml) NETU and (0.01-0.06) µg/ml for PALO. It showed 0.9998

and 0.9999 r2 values for NETU and PALO respectively, that indicates good linearity. Intraday and inter day precision values were indicated as %RSD and %RSD. below 2 showed good precision of developed method. Low LOD and LOQ values indicate sensitivity of proposed method. Accuracy of method was investigated by means of recovery study. Results obtained in range of 96-105% shows good accuracy of developed method. This developed method was also applied to tablet dosage form. All validation parameters were shown in **(Table 8)**. Recovery study data are shown in **(Table 9)**. Robustness study was also performed and low SD indicates that method is robust enough that small changes in method parameter do not affect method responses **(Table 10)**. System suitability parameters were also studied and reported in **(Table 11)**. % Assay obtained for both drugs were 99.814% and 101.52% for NETU and PALO respectively **(Table 12).**

TABLE LEGENDS

Table.1: Composition of the concentration of Calibration set and Validation set

Table.2: Applicability of sample (synthetic mixture)

Table.3: Analysis of validation set by PCR and PLS method

Table.4: Analysis of validation set by CLS method and ILS method

Table.5: Root Mean Square Error of Prediction

Table.6: DoE summary: Critical factors and critical responses

Table.7: Point verification and working point selection

Table.8: Summary of validation parameters for developed method

Table.9: Results of recovery study

Table.10: Results of robustness study

Table 11: System suitability parameters

Table 12: Applicability of developed method

FIGURE LEGENDS

Figure.1: Chemical structure of (A) Palonosetron hydrochloride and (B) Netupitant

Figure.2: zero-order overlay spectra of PALO, NETU and their corresponding binary mixture

Figure.3: Plot of actual vs. Predicted concentration obtained by PCR, PLS, CLS and ILS for Palonosetron hydrochloride and Netupitant.

Figure.4: Plot of Residual concentration vs. Actual concentration of PALO obtained by PCR, PLS, CLS and ILS for Netupitant and Palonosetron HCl

Figure.5: Selection of detection wavelength

Figure.6: Chromatogram of some preliminary trials before applying QbD

Figure.7: Contour plots for Response 1(Retention time of NETU and PALO)

Figure.8: Contour plots for Response 2(Asymmetry of NETU and PALO)

Figure.9: Contour plots for Response 3(Resolution of NETU and PALO)

Figure.10: Desirability graph for selected working point

Figure.11: Design space region for selected response working point

Figure.12: Chromatographic peak for NETU and PALO under optimized condition after applying QbD

Figure.13: Overlay chromatogram for NETU and PALO after applying QbD

Table.1

Calibration set		Validation set	
NETU (µg/ml)	**PALO (µg/ml)**	**NETU (µg/ml)**	**PALO (µg/ml)**
12	0.04	24	0.01
30	0.05	6	0.05
6	0.06	18	0.03
12	0.02	24	0.06
12	0.04	36	0.02
6	0.05	6	0.05
36	0.01	30	0.03
24	0.01	12	0.01
36	0.02	30	0.04
12	0.06	18	0.01
18	0.03	18	0.03
12	0.05	36	0.05
12	0.01		
18	0.03		
30	0.02		
30	0.06		
6	0.04		
12	0.03		
36	0.04		
12	0.06		
24	0.05		
30	0.01		
12	0.04		

Table. 2.

% Assay of Synthetic mixture (±SD) (n=6)		
Method	**Netu(300mg)**	**Palo(0.50mg)**
CLS	100.82±0.0353	99.739±0.0274
ILS	99.94±0.0361	98.858±0.0206
PCR	101.29±0.0319	98.472±0.0235
PLS	100.173±0.0207	100.14±0.0272

Table.3

Added conc. (µg/ml)		Measured conc. (µg/ml)		Recovery (%) by PCR method	
NETU	**PALO**	**NETU**	**PALO**	**NATU**	**PALO**
12	0.04	11.58	0.041	100.62	102.68
30	0.05	30.02	0.049	100.08	100.82
6	0.06	5.63	0.062	99.29	100.64
12	0.02	12.15	0.023	100.59	101.28
6	0.03	6.111	0.028	101.73	98.72
12	0.05	12.02	0.053	100.29	100.19
36	0.01	35.95	0.018	101.49	100.30
24	0.01	24.21	0.014	98.61	99.53
18	0.02	18.02	0.019	99.623	99.28
MEAN				100.26	102.10
% RSD				1.3891	1.1579

Added conc. (µg/ml)		Measured conc. (µg/ml)		Recovery (%) by PLS method	
NETU	**PALO**	**NETU**	**PALO**	**NATU**	**PALO**
12	0.04	11.58	0.041	100.62	102.68
30	0.05	30.02	0.049	100.08	100.82
6	0.06	5.63	0.062	99.29	100.64
12	0.02	12.15	0.023	100.59	99.28
6	0.03	6.111	0.028	101.73	98.72
12	0.05	12.02	0.053	100.29	100.19
36	0.01	35.95	0.018	101.49	100.30
24	0.01	24.21	0.014	98.61	99.53
18	0.02	18.02	0.019	99.623	99.28
MEAN				100.26	99.96
% RSD				1.4391	1.1834

Table.4

Added conc. (µg/ml)		Measured conc. (µg/ml)		Recovery (%) by ILS method	
NETU	**PALO**	**NETU**	**PALO**	**NATU**	**PALO**
12	0.04	11.58	0.041	100.62	102.68
30	0.05	30.02	0.049	100.08	100.82
6	0.06	5.63	0.062	99.294	100.64
12	0.02	12.15	0.023	100.59	99.28
6	0.03	6.11	0.028	101.73	98.72
12	0.05	12.02	0.053	100.29	100.19
36	0.01	35.95	0.018	101.49	100.30
24	0.01	24.28	0.014	99.61	99.53
18	0.02	18.02	0.019	100.62	99.28
MEAN				101.24	100.16
% RSD				1.2748	1.3274

Added conc. (µg/ml)		Measured conc. (µg/ml)		Recovery (%) by CLS method	
NETU	**PALO**	**NETU**	**PALO**	**NATU**	**PALO**
12	0.04	11.58	0.041	100.62	102.68
30	0.05	30.02	0.049	100.08	100.82
6	0.06	5.63	0.062	99.29	100.64
12	0.02	12.15	0.023	100.59	98.28
6	0.03	6.111	0.028	101.73	98.72
12	0.05	12.02	0.053	100.29	100.19
36	0.01	35.95	0.018	101.49	100.30
24	0.01	24.21	0.014	99.61	99.53
18	0.02	18.02	0.019	99.623	99.28
MEAN				102.20	100.13
% RSD				1.3351	1.3237

Table.5

Drug	RMSEP			
NETU	**CLS**	**ILS**	**PLS**	**PCR**
	0.1068	0.1066	0.23460	0.2696
PALO	0.02536	0.02741	0.01371	0.0116

Table.6

CRITICAL FACTORES.		
Factors	**Levels**	**Values**
pH	-1	3.4
	0	3.5
	1	3.6
Flow rate(ml/min)	-1	0.9
	0	1
	1	1.1
Wavelength (nm)	-1	240
	0	241
	1	242

Ratio of buffer solution (ml)	-1	9
	0	9.5
	1	10

Table.7

Optimized solution	Pred. R1 (RT)		Obs. R1 (RT)		Pred.R2 (Asymmetry)		Obs.R2 (Asymmetry)		Pred.R3 (Resolution)		Obs.R3 (Resolution)	
	Palo	Netu	Palo	Netu	Palo	Netu	Palo	Netu	Palo	Netu	Palo	Netu
pH 3.5, F.R 1,Wavelength 241, buffer ratio 10ml.	3.31	5.83	3.52	6.33	1.548	1.76	1.690	1.63	3.73	12.15	3.27	12.56
pH 3.50, F.R 0.9,wavelength 242.7, buffer ratio 10ml.	3.35	5.81	3.50	6.028	1.589	1.73	1.791	1.74	3.45	12.28	3.34	12.38
pH 3.5, F.R 0.9,wavelength 241, buffer ratio 9 ml.	3.38	5.73	3.53	6.135	1.545	1.78	1.754	1.72	3.67	12.23	3.55	12.49
pH 3.4, F.R 0.95,wavelength 242.8, buffer ratio 9.5ml.	3.31	5.79	3.57	6.282	1.571	1.71	1.601	1.63	2.077	11.68	3.13	12.73
pH 3.6, F.R 1, wavelength 241, buffer ratio 10ml.	4.02	5.92	3.97	7.310	1.557	1.77	1.579	1.68	2.518	12.46	3.43	11.39

Table.8

PARAMETER	NETU	PALO
Linearity range(µg/)	6-36	0.01-0.06
Correlation coefficient	0.9998	0.9999
Slop	25.30	23.05
Intercept	12.12	17.52
Intraday precision(%RSD)	1.4368	1.2872
Interday precision(%RSD)	1.2256	1.1482
LOD (µg/ml)	0.7435	0.5594
LOQ (µg/ml)	0.5310	0.4591

Table.9

%spiking	C actual (µg/ml)		C added (µg/ml)		C recover (µg/ml)		% recovery	
	Netu	Netu	Netu	Palo	Netu	Palo	Netu	Palo
80	12	0.02	9.6	0.016	8.6587	0.0169	101.12±0.53	99.9±0.583
100	12	0.02	12	0.02	12.131	0.0189	101.31±0.82	100.4±0.417
120	12	0.02	14.4	0.04	18.799	0.0369	102.52±0.58	99.63±0.574

Table.10

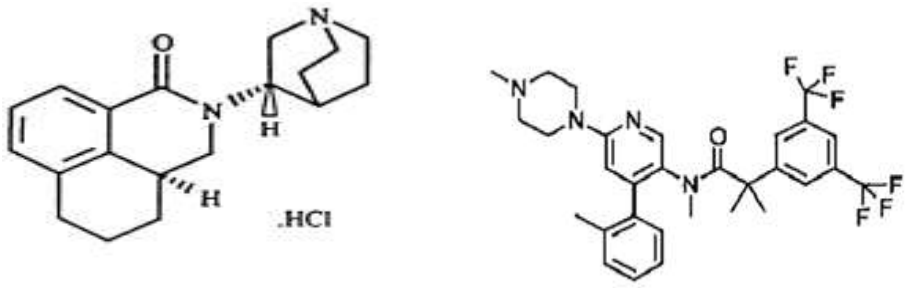
Factor	Retention time (min)		Peak Area (mV.s)	
	NETU	PALO	NETU	PALO
A. Wavelength				
239 nm	6.025	3.507	761.462	122.205
241 nm	6.373	3.575	877.675	278.363
243 nm	6.389	3.582	778.154	127.467
Mean ±SD	6.375±0.0271	3.531±0.0231	805.564 ±0.632	176.763±0.5329
B. Flow rate				
0.9	5.767	3.317	798.662	126.566
1.0	6.301	3.515	873.548	278.569
1.1	6.325	3.638	647.393	120.849
Mean ±SD	6.357±0.0275	3.518±0.0235	773.328±0.3771	175.201±0.5616
	C. pH of buffer			
3.0	6.284	3.507	807.397	183.641
3.5	6.325	3.582	784.664	173.861
4.0	7.621	3.619	750.621	177.349
Mean± SD	6.336±0.0235	3.552±0.0493	645.753±0.475	206.5606±0.829

Table.11

PARAMETER	DATA OBTAINED (n=6)	
	NETUPITANT	PALONOSETRON. HCl
Retention time (min) ± SD	6.036±0.0423	3.526 ±0.0539
Theoretical plate ± SD	10470±126.566	8936±741.815
Asymmetry factor ± SD	1.240±0.0628	1.526±0.0361
Resolution ± SD	5.618±0.173	3.288±0.384

Table.12

% Assay of Synthetic mixture (±SD) (n=6)		
METHOD	**NETU (300mg)**	**PALO (0.50mg)**
RP-HPLC	101.52± 0.253	99.814± 0.574



(A) **(B)**

Figure.1

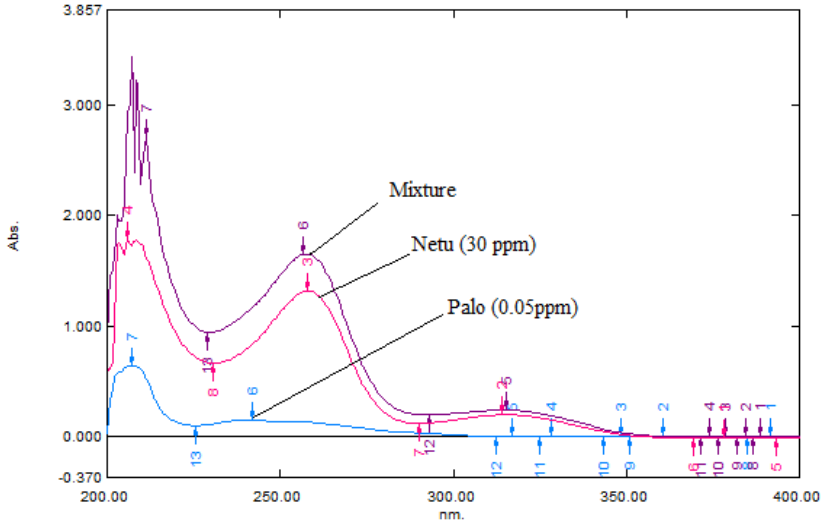
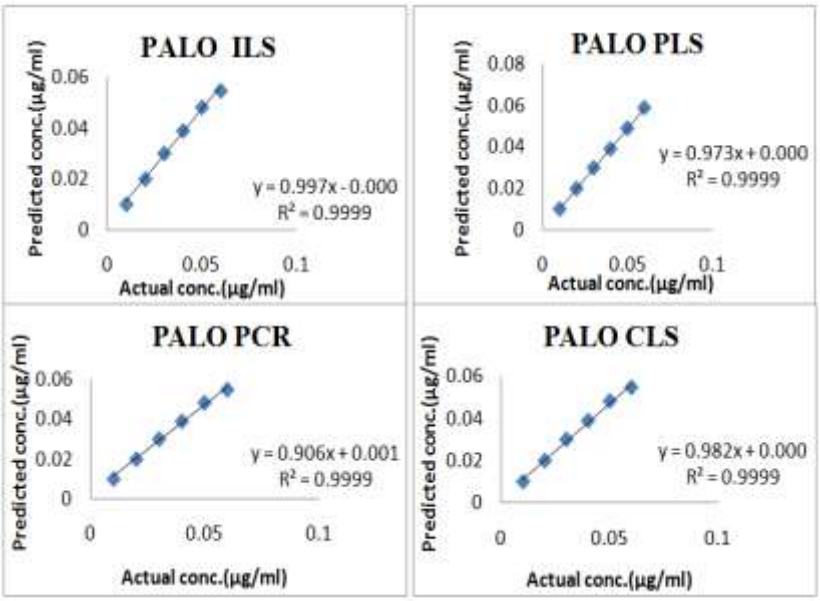


Figure.2



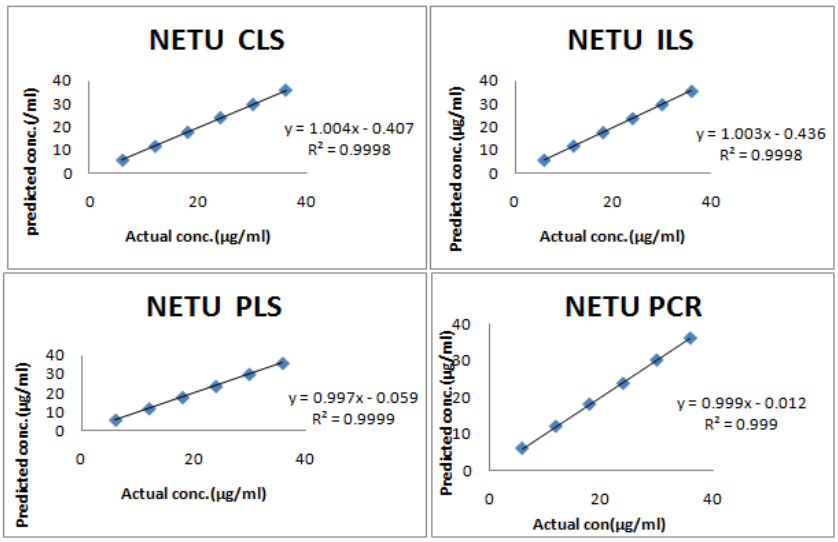
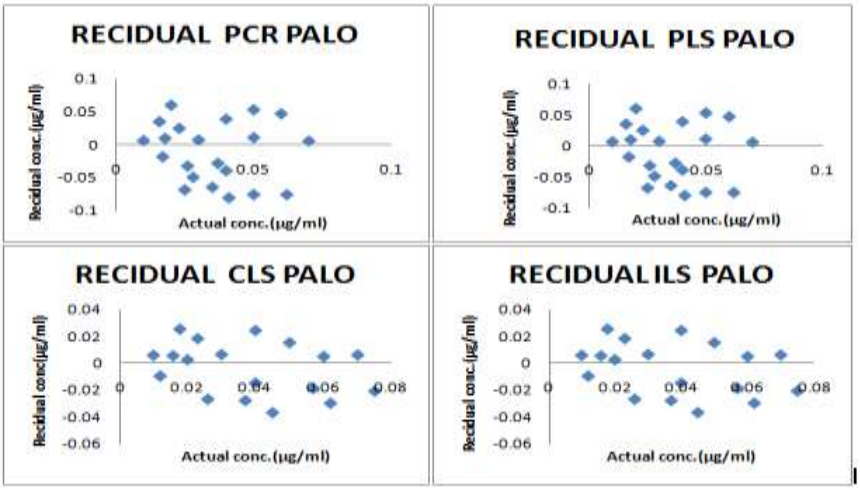


Figure.3



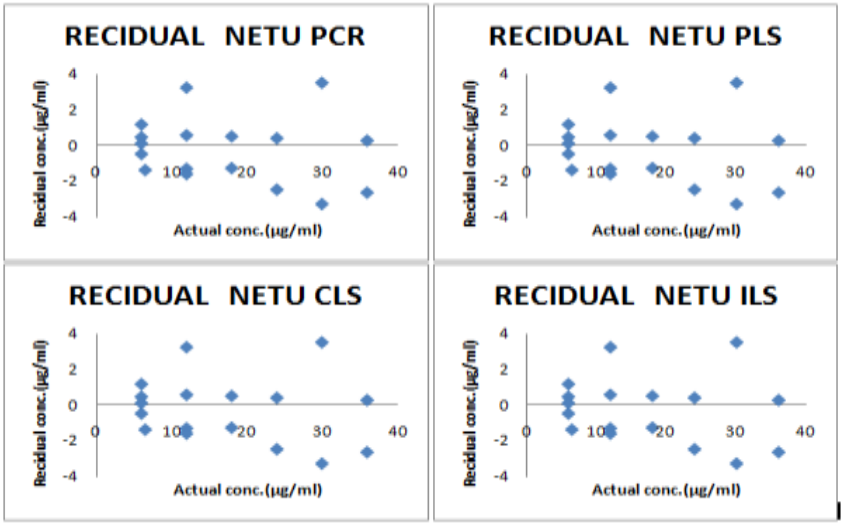


Figure.4

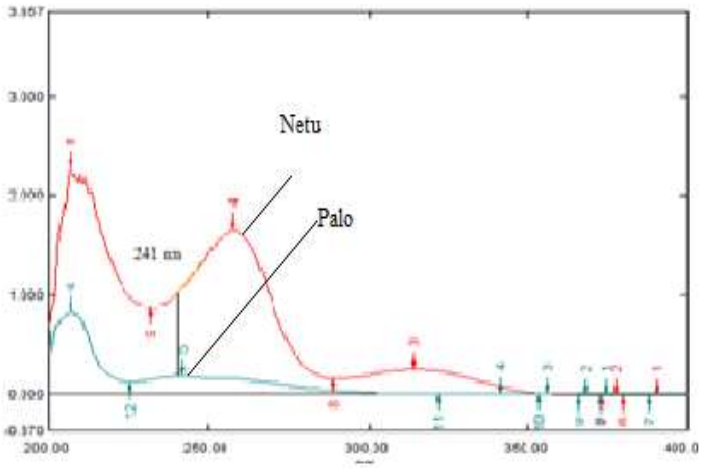


Figure.5

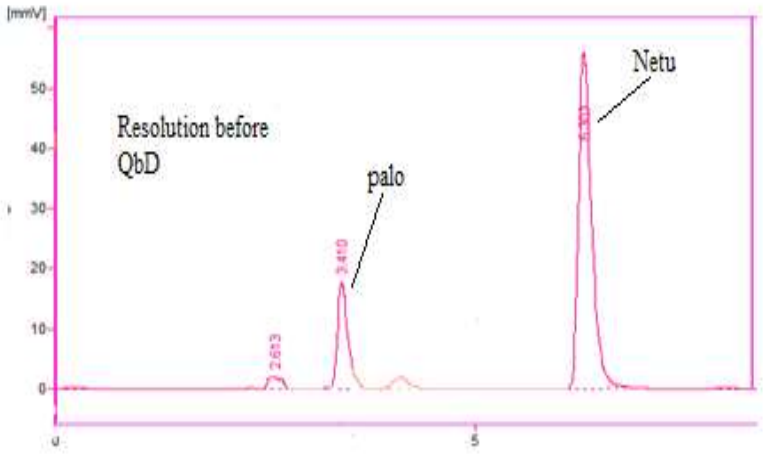


Figure.6

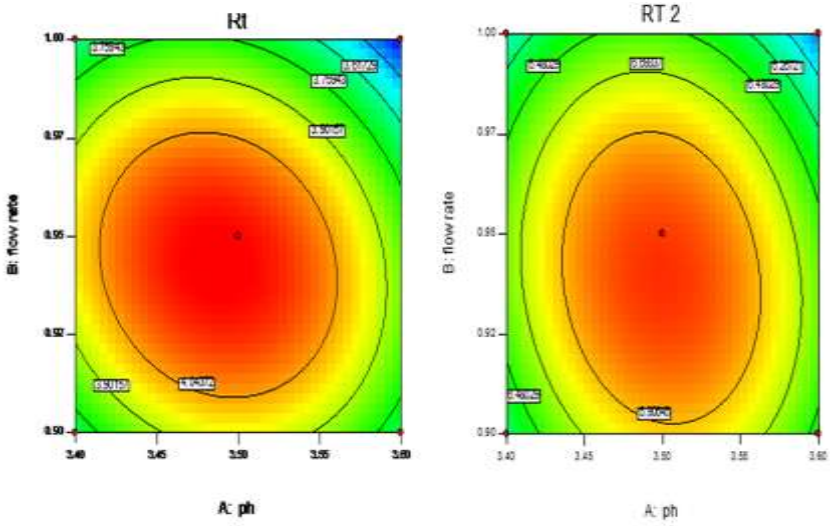


Figure.7

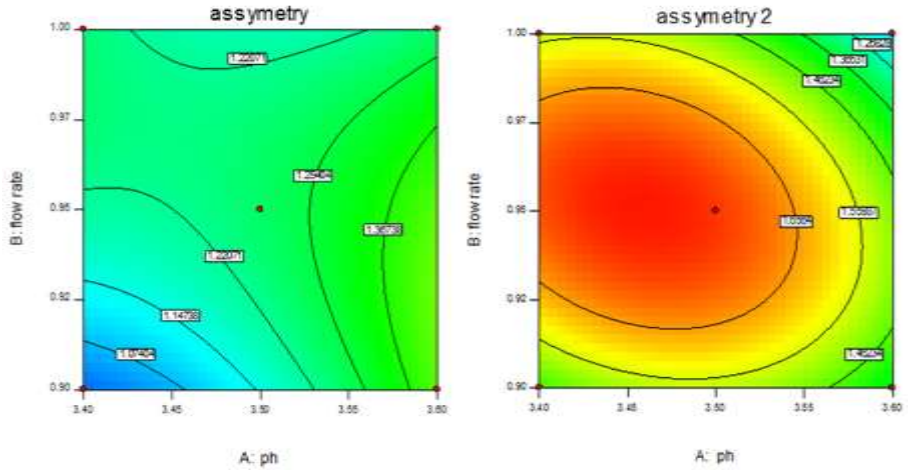


Figure.8

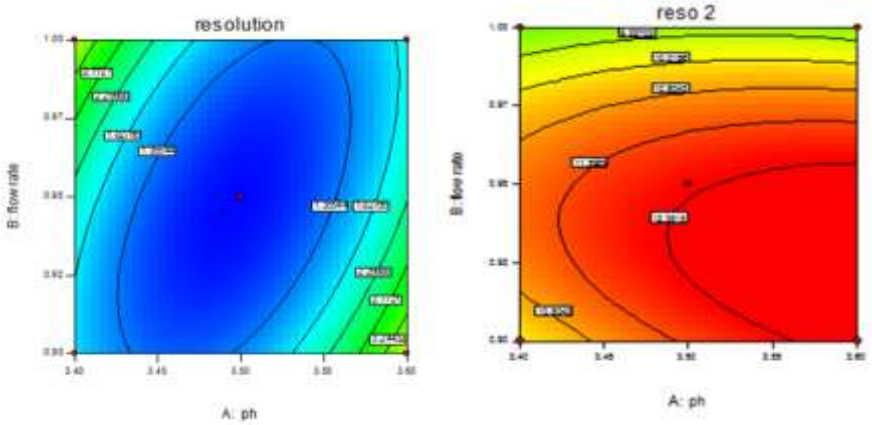


Figure.9

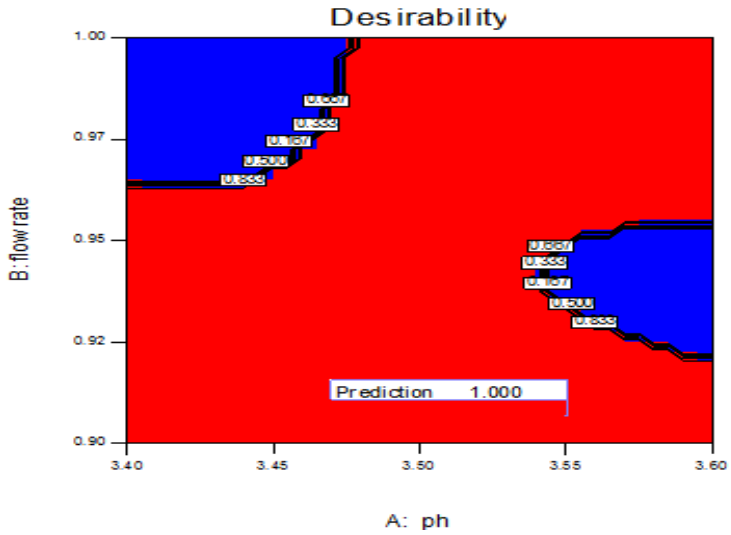


Figure.10

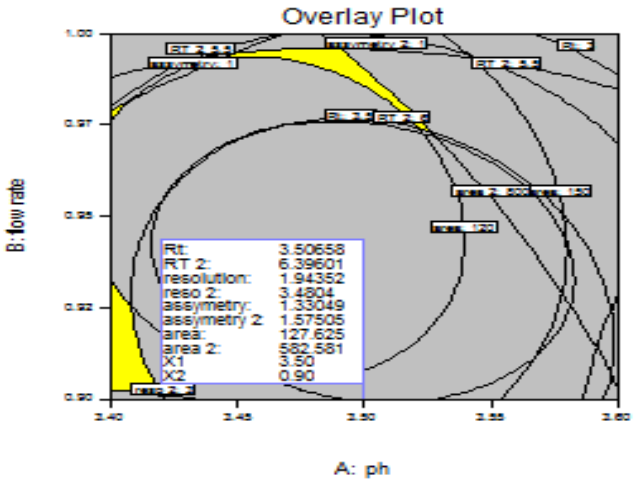


Figure.11

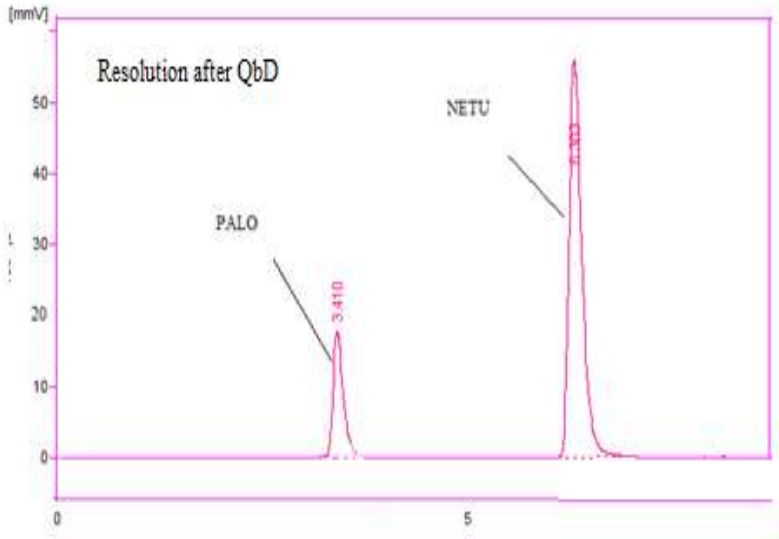


Figure.12

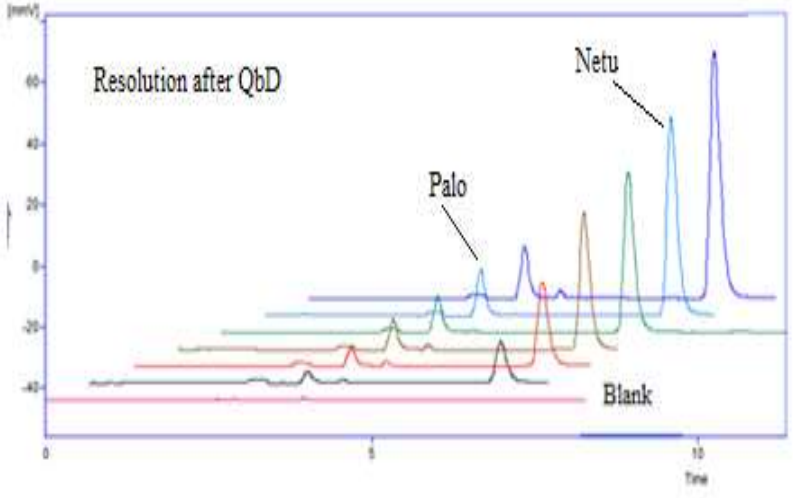


Figure.13

CONCLUSION

Chemometrics assisted and an innovative quality by design approach has been applied for development of RP-HPLC methods are an effective tool for simultaneous quantification of PALO and NETU which offers striking advantage in terms of time required for analysis, simplicity, accuracy, and precision. All the methods are found to be applicable for synthetic mixture. The methods were developed, validated and satisfactory results were produced.

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