Journal of Chromatography
Separation Techniques
ISSN: 2157-7064

Seetharaman and Lakshmi, J Chromatograph Separat Techniq 2014, 5:3
DOI: 10.4172/2157-7064.1000229

Research Article Open Access

Development and Validation of a Reverse Phase Ultra Performance Liquid Chromatographic Method for Simultaneous Estimation of Nebivolol and Valsartan in Pharmaceutical Capsule Formulation

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Abstract

The present study describes the development and validation of sensitive, novel Ultra Performance Liquid Chromatographic technique to simultaneously evaluate Nebivolol and Valsartan in commercial pharmaceutical capsule formulation. The technique was performed utilizing thermo C_{18} (4.6 mm×50 mm, 1.9 µm) column having a mobile phase comprising of 10 mM ammonium dihydrogen phosphate pH adjusted to 3.00 ± 0.02 with dilute orthophosphoric acid as buffer, with a ratio of buffer: acetonitrile 60:40 (v/v) and with a flow rate of 0.4 mL min^{-1}. The eluted components were monitored out at 220 nm utilizing a photo diode array detector. The retention times for NBL and VST were 0.48 and 0.83 min respectively. The developed Ultra Performance Liquid Chromatographic method was validated according to ICH guidelines to confirm specificity, linearity, accuracy and precision. The homoscedasticity of the variances and lack of fit in the evaluation of linearity was established using the Cochran's C test and F-test respectively. The repeatability variances and time different intermediate variances were assessed simultaneously. The time different variances expressed as percentage relative standard deviations (%RSD) and accuracy were well within the limits as prescribed by ICH. In order to designate suitability in the experimental design approach, a robustness test was carried out. To assess robustness 3 aspects were taken into consideration, namely, proportion of flow rate, proportion of acetonitrile in mobile phase and pH; all the three factors have no significant effect on response (assay). Experimental design based robustness with the aid of Full Factorial design (FFD) provided an effective way to simultaneously asses Nebivolol and Valsartan. This method was successfully used to analyze fixed dose capsule samples of Nebivolol as well as Valsartan and can be utilized for regular lab investigation of Nebivolol and Valsartan in capsules.

Keywords: Ultra Performance Liquid Chromatography; Experimental design; Robustness; Nebivolol; Valsartan

Introduction

Nebivolol (NBL) chemically known as α,α-[iminobis(methylene)] bis[6-flouro-3,4-dihydro-2H-1-benzopy-ran-2-methanol] and is shown in Figure 1a [1]. Its empirical formula is $C_{22}H_{25}F_2NO_4$. Nebivolol, an antihypertensive drug is a competitive and cardio selective beta blocker with limited vasodilating properties, probably due to an interaction with the l-arginine/nitric oxide pathway [2].

Valsartan (VST), chemically known as N-(1-oxopentyl)-N-[[2' (1H-tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl]-L-valine and is shown in Figure 1b [1]. Its empirical formula is $C_{24}H_{29}N_5O_3$. VST is an active non peptide angiotensin II receptor antagonist VST displaces angiotensin II from the AT_1 receptor and produces its blood pressure lowering effects [2].

The combination therapy of NBL and VST is indicated for the potential treatment of hypertension, to lower blood pressure. The fixed dose combination of nebivolol and valsartan proved to be statistically sound in reducing the diastolic blood pressure against the highest approved doses of both NBL alone (40 mg) and VST alone [3].

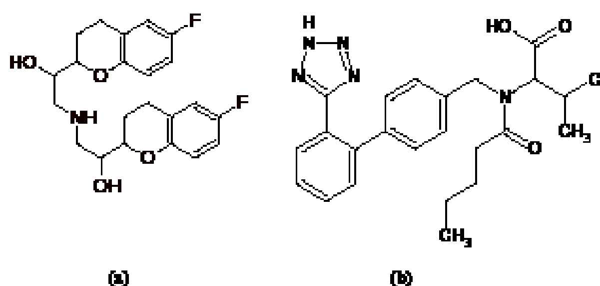

Figure 1: Chemical Structure of (a) NBL (b) VST.

The literature reveals few Ultra Performance Liquid Chromatographic (UPLC) techniques were documented to establish VST [4-5] individually or with some other drugs in pharmaceuticals, the review further reveals that no UPLC methods were reported for the estimation of NBL in pharmaceuticals and biological samples. To date, no UPLC technique is reported to concurrently determine NBL and VST in medicinal dose as capsules with short run time. Therefore it is felt necessary to develop a fast liquid chromatographic method with short analysis time.

As per the review made on the literature few HPLC methods [6-12] were reported for the estimation of NBL and VST. The amount of solvent and the time required for the estimation of selected drugs by the reported HPLC methods were more. The technique of UPLC is more

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Received May 21, 2014; **Accepted** June 30, 2014; **Published** July 18, 2014

Citation: Seetharaman R, Lakshmi KS (2014) Development and Validation of a Reverse Phase Ultra Performance Liquid Chromatographic Method for Simultaneous Estimation of Nebivolol and Valsartan in Pharmaceutical Capsule Formulation. J Chromatograph Separat Techniq 5: 229. doi:10.4172/2157-7064.1000229

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advantageous than HPLC with the amount of solvent and time required for analysis. The capability and quickness of analysis is becoming more significant in the field of pharmaceutical, toxicological and clinical studies [13-15].

A significant reduction in division period and solvent utilization is favoured by UPLC. Research papers reveal that UPLC structure permits approximately nine times reduction in period for investigation in comparison to the conventional HPLC structure utilizing 5 µm unit dimension analytical columns, and approximately 3 times reduction in investigation period when compared to 3 µm unit dimension analytical columns with no concession on the whole division [13-15].

Investigational method was utilized for the substantiation to assess the strength of the method. The objective of this paper is to address the robustness of UPLC assay method and to explore the significant factors from a FFD. It also provides an effective case study on the experimental design application on the assay method of a pharmaceutical dosage form.

Experimental

Instrumentation and apparatus

The UPLC structure utilized for method development and validation happens to be Thermo accela™ equipped with 1050 quaternary pump auto sampler and a photodiode array (PDA) detector. The UPLC PDA detector had 10 mm, 2 µL Light Pipe flow cell. The yield of the detector was documented and developed utilizing Chrome quest software version 5.0, Sonicator (PCI bath sonicator) was utilized for degassing of movable stage as well as sonication of the liquids prepared.

Software

The investigational method and data examination were done by utilizing Unscrambler X edition 10.1; other statistical calculation for the analysis was performed by using Microsoft Excel 2007 software (Microsoft, USA) and Sys Stat 2013 trial version (Sys Stat).

Chemicals, pharmaceutical preparation and reagents

Reference norms of NBL and VST were kindly gifted by Ideal Analytical and Research Institution (Puducherry, India) with stated purity of 99.85% and 99.94%, correspondingly. All the values were used as obtained. Market sample of Nebicard –V capsules claiming 5 mg of NBL and 80 mg of VST were obtained from retail drug store in Chennai. HPLC quality water, acetonitrile, analytical reagent category of orthophosphoric acid was obtained from Rankem, India.

Conditions for chromatographic methods

The chromatographic partition was done on a Thermo C_{18} 50×2.1, 1.9 µm particle size. The mobile phase comprises of mixture of 10 mM ammonium dihydrogen phosphate buffer (pH adjusted to 3.00 with dilute orthophosphoric acid) and acetonitrile to the proportion of 60:40 (v/v). The flow rate and injection volume was 0.4 mL min^{-1} and 1 µL respectively. The column warmth was ambient and the zeniths were observed at 220 nm.

Preparation of standard solutions

Stock standard mixtures containing NBL and VST (100 µg mL^{-1} of NBL and 1600 µg mL^{-1} of VST) were produced by mixing suitable quantities of the compounds in mobile phase. Working mixtures 10 µg mL^{-1} of NBL, 160 µg mL^{-1} of VST were produced from the fore said stock mixture in mobile phase for test inference.

Analytical method validation

Solution stability: The key aspect of establishing solution stability is to favor the analytical method to employ auto samplers in the estimation of drugs, as the prepared solutions is allowed to stand overnight and even more than that. The stability of the NBL and VST was assessed by leaving the sample and standard solution in a tightly capped standard flasks at room temperature for 12 hours during which they were assessed for assay at 6 hours intervals. The amount of NBL and VST was calculated for evaluation of solution stability.

System suitability: So as to confirm the system functioning, system appropriateness parameters were measured. With six repeated additions of customary arrangements, system accuracy was decided. Every significant feature together with capability aspect, peak resolution, plus theoretical plate number was calculated.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, [16]. Based on the sample preparation procedure, an investigative placebo solution (including all the inactive substances other than NBL as well as VST) was produced and injected. With the help of this developed method, the interference of these excipients is analyzed for a mixture of inactive ingredients, commercial pharmaceutical preparations including NBL and VST and standard solutions.

Linearity of the calibration line: A standard stock solution claiming 100 µg mL^{-1} and 1600 µg mL^{-1} of NBL and VST respectively were prepared. Seven standard solutions were prepared by serial dilution from the stock solution of NBL and VST. An aliquot of 1 µL of each of the calibration solution was injected in the UPLC system. Linearity was performed between 70% and 130% of normal strength utilizing seven calibration intensities (70%, 80%, 90%, 100%, 110%, 120% and 130%) for all the compounds. The linearity was done in triplicate and for three days. Cochrans C test [17] was applied to verify the homogeneity of variances (homoscedasciticity) of the residuals along the line of regression. As the homoscedasticity of the calibration line was satisfactory for the regression line of three analytes the slope and intercept were calculated with their 95% confidence intervals employing Ordinary Least squares. The linearity was assessed visually by observation of calibration line, and statistically by employing F-test [18] for Lack of fit.

Precision: Precision was examined utilizing the proposed method for six genuine commercial samples of Nebicard V. Repeatability and Intermediate precision were evaluated simultaneously [16]. Precision was assessed by under taking six self-determining evaluations NBL and VST at 100% of the target concentration of each compound. The amount of NBL and VST was evaluated against a competent reference benchmark. The assessment was done in duplicate for three days.

Accuracy: With the standard addition method (spiking), revival trials were carried out for verifying the correctness of the proposed technique. 80%, 100% and 120% are the three different standard levels added to pre-analyzed capsule samples. The concentration of each drug injected in UPLC was 8, 10, 12 µg mL^{-1} of NBL and 128.00 µg mL^{-1} , 160.00 µg mL^{-1} , 192.00 µg mL^{-1} of VST. The solution for accuracy is prepared in triplicate and assessed. The procedure was done in triplicate and for three ensuing days. The proportion recovery of NBL and VST at every stage was assessed against competent reference benchmark.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during

normal usage [16]. The robustness was assessed and evaluated by experimental design. The study of robustness with the aid of factorial design [18] involves varying parameters simultaneously rather than one at a time can be more efficient, and allows the effects between parameters to be observed. Comparative, response surface modelling, regression modelling and screening are the four common types of multivariate experimental design based approaches. The focus of the propose work was on screening as it is the most appropriate design for robustness studies. Screening design is an efficient way to identify the critical factors that affect robustness. The most common type of screening experimental design are full factorial, fractional and Plankett Burmann designs.

Full factorial design: Full factorial investigational plan with two or more features wherein all the stages of every feature is connected. It could be further referred to a fully-crossed plan. A full factorial experimental design permits one to understand the impact of every feature on the reaction variables and the impacts of interactions among factors. A common full factorial design is one with all factors set at two levels each, a high and low value The quantity of trials to be carried out is a role of the number of factors and the number of level for every factor. If there are k factors, each at two levels, a full factorial then has 2^k runs. In other words, using four factors, there would be 2^4 or 16 design points or run [18].

Determining of factors: The factors assessed are the flow ratio (A), proportion of acetonitrile (B) as well as pH (C). The selected factors are studied at two levels symmetrically situated around the nominal one. Table 1 illustrate the selected factors and the range investigated

Between two and five center replications are commonly done to ascertain the investigational fault variance and to check the analytical soundness of the method (B). A complete factor factorial plan was used in robustness testing for the selected factors not exceeding three levels (−1, 0, +1); the plan employed in robustness tests of NBL as well as VST was a full factorial plan. The investigational domains of the particular variables plus the equivalent reactions are documented in Table 4.

Every one of the trials was carried out in an arbitrary manner to reduce the impacts of unrestrained variables which might bring in a prejudice on the dimensions. Three duplicates of the core features were carried out to assess the investigational fault. The notation for a linear regression method containing three predictor variables with interactions is

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \varepsilon \quad (1)$$

Wherein Y is the reaction of the model, β is the regression coefficient and X_1, X_2 and X_3 symbolize features A, B and C correspondingly, β_1, β_2 and β_3 are the impact coefficients for the main effects of factors A, B as well as C, correspondingly. β_{12}, β_{13} and β_{23} are the impact coefficients for the AB, AC as well as BC interactions, whereas β_{123} symbolizes the ABC interface.

The equation for the regression method is very suitable, particularly if there is a huge amount of higher order interactions existing.

Factor	Levels		
	(-)	Nominal (0)	(+)
(A) Flow rate (μL min $^{-1}$)	360	400	440
(B) Acetonitrile (%)	36	40	44
(C) pH	2.80	3.00	3.20

Table 1: Selected factors and range investigated during robustness testing.

Preparation of sample solution: The method was applied for estimation of NBL and VST in pharmaceutical capsules. 20 capsules of Nebicard - V were taken; their average nett content was established and powdered to a good homogenous dust. A precisely measured amount of the powder corresponding to one capsule (5 mg of NBL and 80 mg of VST) was kept in a 50 mL volumetric flask. To this flask, approximately 35 mL of mobile phase was included and sonicated for a time of 5 min in a sonicator, later thinned to the mark with mobile phase and blended thoroughly to obtain sample stock solution. The sample stock solution was strained via a whatmann no. 41 filter paper and the remains was saved following the rejecting the initial small number of millilitres. One millilitre of the filtrate was poured into a 10 mL volumetric flask, thinned to capacity with mobile phase and blended thoroughly. The sample solution was prepared in triplicate and the amount of NBL and VST were analysed in accordance with the proposed method.

Results and Discussions

Method development and optimization

The main objective of the proposed method is to provide a fast and reliable analytical procedure for the estimation of NBL and VST. To obtain the best chromatographic condition, different columns like C_8, C_{18} and the mobile phase composed of Ammonium dihydrogen phosphate buffer and organic modifier like methanol and acetonitrile and the pH ranged from 2.6 to 3.8 was used as a starting point. The best chromatographic condition was achieved with a C_{18} Column with a mobile phase comprising 10 mM ammonium dihydrogen phosphate pH adjusted to 3.00 ± 0.02 with dilute orthophosphoric acid as buffer, with a ratio of buffer: acetonitrile 60:40 (v/v) and with a flow rate of 0.4 mL min $^{-1}$. The detection was monitored out at 220 nm. The optimum wavelength for detection was 220 nm at which detector responses were more appropriate for the selected drugs. Under the proposed UPLC chromatographic parameters, NBL and VST were well separated and their analogous peaks were distinctly developed at feasible retention times.

The reputations of organic modifier (concentration) such as acetonitrile and pH were carefully studied. 40 volumes of acetonitrile with 60 volumes of buffer at a flow rate of 0.4 mL min $^{-1}$ gave good separation and reduced retention time of NBL and VST, while the pH of 3.00 ± 0.02 gave good resolution and peak shape.

Solution stability

The variability obtained in the estimation of NBL and VST was within ± 2% during mobile phase and solution stability experiments, which confirmed solution and mobile phase stability up to 12 hours for assay (Table 2).

Results of Validation

Specificity: The chromatogram obtained for the placebo solution did not show any peak (Figure 2) at the retention time of NBL as well as VST, while the chromatogram of the placebo solution spiked with NBL and VST showed well separated peak (Figure 3) of sample which indicates the specificity of the proposed method.

System suitability: The percentage R.S.D. of retention period plus peak region of NBL and VST of six duplicate injections of standard solution was lower than 2.0%. The findings of structure accuracy are illustrated in Table 3. The % R.S.D values were for duplicate injections which that the structure is accurate. Findings of other system appropriateness strictures like capacity factor, resolution as well as hypothetical plates are illustrated in Table 3 and were within the specified limits.

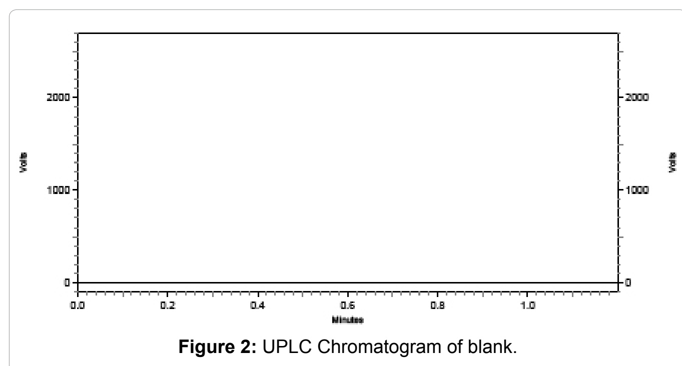

Figure 2: UPLC Chromatogram of blank.

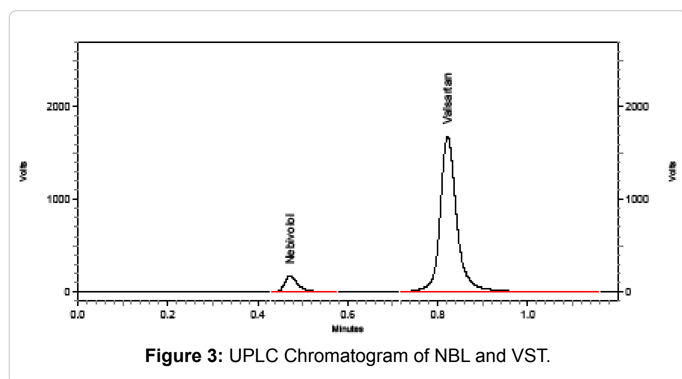

Figure 3: UPLC Chromatogram of NBL and VST.

Analyte	Initial % recovery	6 h		12 h	
		% recovery	D_a	% recovery	D_a
NBL	99.98	98.56	0.01	97.82	0.02
VST	99.95	98.63	0.01	98.01	0.02

[Da] Percent difference calculated by the difference between two values divided by the average of the two values

Table 2: Results of solution stability.

Parameters	NBL	VST
t_R	0.47	0.82
% RSD of t_R for 6 Injections	0.63	0.66
N	11236	18226
R_s	5.98	
A_s	0.56	0.39

t_R retention time, N Number of theoretical plates, R_s resolution factor, A_s Peak asymmetry

Table 3: Results of system suitability parameters for the estimation of NBL and VST.

Precision: The repeatability variances for NBL and VST at 100% concentration level were 0.28 and 0.27 respectively. The time different intermediate precision variances for the same concentration level were 0.26 and 0.42 for NBL and VST respectively. The corresponding pooled variance and the % RSD were 0.54 and 0.73 for NBL and 0.69 and 0.84 for VST respectively. The % RSD values obtained imply that the precision values obtained were satisfactory.

Linearity: Cochrans C test was employed at first before the regression analysis to verify homoscedascity of the calibration standards and was found to be satisfactory. The C_{calc} values were 0.198 and 0.195 for NBL and VST respectively. The critical value [17] is, $C_{tab(\alpha=0.05;k=7,\ n=9)}$ = 0.338. The results of the cochrans's C test prove that the variances of the calibration standards were homoscedastic.

The regression lines were evaluated by ordinary least squares. The regression equation of the calibration lines for NBL and VST were Area $_{NBL}$ = 27288C_{NBL} (µg mL^{-1}) + 1050 and Area $_{VST}$ = 23585 C_{VST} (µg mL^{-1}) + 917 respectively and the corresponding values of slopes and intercept at their 95% confidence limits were 27288 ± 2.45 and 1050 ± 77.18 for NBL and 23585 ± 0.24 and 1117 ± 70.36 for VST. The correlation coefficients were 0.9995 and 0.9999 respectively.

The variances of residuals from the calibration data of NBL and VST was evaluated using ANOVA for lack of fit test [17].

The results of the lack of fit test for residuals of the calibration data of NBL and VST were Fcalc = 1.84 and 0.89 respectively. The test values obtained were less that the critical value, $F_{tab\ (\alpha=0.05 df1=6,\ df2=56)}$ = 2.265.

The peak area was established linear between 70% and 130% of target concentration. The homoscedasticity of the calibration standards was satisfactory. For every compound the connected coefficient was more than 0.9990 along with cochran's and lack of fit test proves linearity.

Accuracy: The quantity claimed was contained by ± 3% of quantity included that showed that the technique is precise and in addition exclude the intrusion owing to excipients existing in capsules. The mean percentage recovery for NBL at 80%, 100% and 120% concentration levels were 99.6% ± 0.49, 100.03% ± 0.64 and 99.97% ± 0.39. The % recovery range was 99.37 -100.49% and the mean recovery for all the concentration levels at 95% confidence limits were 99.89% ± 0.38.

The % recoveries for VST at the same concentration levels were 99.91% ± 0.36, 100.03% ± 0.64 and 99.69% ± 0.29. The percentage recovery range was 98.55-100.31% and the mean recovery for all the concentration levels at 95% confidence limits were 99.56% ± 0.37.

Robustness: The method was authenticated by the examination of variance (ANOVA) using Unscrambler X. The numerical examination illustrated (Table 5) that the method symbolizes the occurrence excellently and the difference of the reaction was accurately connected to the difference of the features.

The ANOVA chart obtained is a synopsis of the importance of the worldwide method. If p-value for the worldwide method is lesser than 0.05, it discloses the method is noteworthy at 5% level. That is a lower P-value the additionally important is the method. As the p values obtained are more than 0.05 null hypotheses Ho is accepted [17]. The effect summary is reported in Table 5 which offers an outline of the importance of every impact for all reactions.

Run	Design			Response	
	Coded factors[a]			Assay (%)	
	Factor – A Flow rate (µL min^{-1})	Factor – B Acenonitrile (%)	Factor – C pH	NBL	VST
1	-1	-1	1	98.23	98.95
2	-1	1	-1	99.56	99.61
3	-1	-1	-1	99.52	99.54
4	0	0	0	99.89	98.73
5	1	-1	-1	100.56	99.52
6	1	1	-1	100.16	98.56
7	1	1	1	99.61	98.93
8	0	0	0	99.28	99.86
9	-1	1	1	99.47	99.85
10	0	0	0	98.66	98.23
11	1	-1	1	98.36	99.56

[*]Randomized.

[a]The low, middle (central) and high level of the factors were designated as (-1), (0) and (1), respectively.

Table 4: 2^3 Full factorial experimental plans for robustness testing and obtained responses[*].

Analyte	Variables	Sum of squares	Degree of freedom	Mean square	F value	P value	Effect value	Significance
NBL	A	1.103	2	0.551	5.010	0.11	0.74	NS*
	B	1.103	2	0.551	5.010	0.11	0.74	
	C	0.637	2	0.318	2.800	0.18	-0.56	
	AB	0.002	4	0.001	0.000	0.92	0.03	
	AC	0.007	4	0.002	0.030	0.87	0.05	
	BC	0.300	4	0.075	1.360	0.32	0.38	
	ABC	0.160	8	0.020	0.720	0.45	-0.28	
	Pure error	0.659	27					
	corrected total		53					
VST	A	0.016	2	0.008	0.126	0.74	-0.09	NS*
	B	0.027	2	0.013	0.207	0.67	-0.11	
	C	0.151	2	0.076	1.185	0.35	-0.27	
	AB	0.039	4	0.010	0.307	0.61	-0.14	
	AC	0.259	4	0.065	2.031	0.24	0.36	
	BC	0.594	4	0.149	4.654	0.11	-0.54	
	ABC	0.500	8	0.063	3.917	0.14	-0.50	
	Pure error	0.383	27					
	corrected total		53					

* Not significant

Table 5: Results of ANOVA and effect summary.

Product name (Composition)	Manufacturer	Percentage found	
		NBL	VST
Nebicard - V (Nebivolol 5 mg and Valsartan 80 mg)	Torrent pharmaceuticals	99.63*	99.86*

*average of six readings

Table 6: Results of Analysis of Marketed formulations.

The regression equation model for NBL, VST is in equation 2 and 3

$$Y_{NBL} = 99.39+0.37X_1 +0.37X_2-0.28X_3+0.01\ X_1X_2+0.02\ X_1X_3+0.19\ X_2X_3-0.14\ X_1X_2X_3 \quad (2)$$

$$Y_{VAL} = 99.28-0.04X_1 -0.05X_2-0.13X_3-0.07\ X_1X_2+0.18\ X_1X_3-0.27\ X_2X_3-0.25\ X_1X_2X_3 \quad (3)$$

In conclusion, by examining the ANOVA results confirms that Y_{NBL} and Y_{VST} are robust for all the three factors.

Results of analysis in capsule formulation: The results obtained with the analysis of marketed capsule formulation was carried out and the recovered amount each drug component were expressed as percentage amount of label claim. The results are presented in Table 6, which shows that in all the selected formulations for the study NBL and VST ranged between 99.45 to 99.69%, and 99.63 to 99.86% respectively. These values comply with the assay specifications for active drugs in the USP pharmacopeia (90.0–110.0%) [19], which are required to be met by most drug formulations.

Conclusions

The selected analytes such as NBL and VST have been simultaneously analyzed in pharmaceutical formulation (capsules) with UPLC. The entire run time happened to be 1 min, wherein the two peaks NBL and VST were well separated. The proposed rapid UPLC method had been assessed on the linearity, accuracy, precision, specificity and robustness and established to be suitable and effectual in the quality assessment of NBL as well as VST in Pharmaceutical capsule hence can be used in QC laboratories for the estimation of NBL and VST. All the validation results were comparable with the already reported HPLC method. The developed UPLC method is more advantageous when compared to the reported HPLC method [6-12] in terms of analysis time, cost and consumption of solvents, sensitivity etc. The finding of the attempt reveal the advantage of utilizing experimental design based robustness study in method validation.

Acknowledgement

The authors are highly grateful to the management of SRM College of Pharmacy, SRM University, Chennai and Ideal Analytical and Research Institution, Villianur, Puducherry, for offering conveniences to conduct the study.

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