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Quality by design (QBD) approach to develop stability indicating RP-HPLC method development for naproxen and pantoprazole

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***Abstract*---**Background and Objectives: As per requisition of current regulatory requirements, simple, rapid and sensitive method by 3^3 factorial QbD approach was established and validated for Naproxen (NPX) and Pantoprazole (PPR) by RP-HPLC. Method: A simple RP-HPLC method has been developed and validated with different parameters such as linearity, precision, repeatability, LOD, LOQ, accuracy as per International Conference for Harmonisation guidelines (Q2R1). Statistical data analysis was done for data obtained from different aliquots Runs on Agilent Tech. Gradient System with Auto injector, UV (DAD) & Gradient Detector. Results: Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 100mm; 2.5µm), a 20µl injection loop and UV730D Absorbance detector at 239 nm wave length and running chemstation 10.1 software and drugs along with

degradants were separated via Methanol: (0.1% OPA) Water (75:25) of pH 3 as mobile phase setting flow rate 0.7 ml/min at ambient temperature. The developed method was found linear over the concentration range of 10-50 µg/ml for NPX and 4-20 µg/ml for PPR while detection and quantitation limit were found to be 0.2375 (ug/mL) and 0.7199 (ug/mL) for NPX and 0.1028 (ug/mL) and 0.3059 (ug/mL) PPR. Conclusion: There are no interfering peaks underperformed degradation conditions. Therefore, a sensitive, robust, accurate and stability indicating method was developed with high degree of practical utility.

***Keywords*---**naproxen, pantoprazole, RP-HPLC, stability study, method development, validation.

Introduction

The concept of "Quality by Design" (QbD) was defined as an approach which covers a better scientific understanding of critical process and product qualities, designing controls and tests based on the scientific limits of understanding during the development phase and using the knowledge obtained during the life-cycle of the product to work on a constant improvement environment. QbD describes a pharmaceutical development approach referring to formulation design and development and manufacturing processes to maintain the prescribed product quality. Guidelines and mathematical models are used to ensure the establishment and use of the knowledge on the subject in an independent and integrated way [1-3].

Naproxen chemically, (2*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid; Figure 1 Naproxen is a propionic acid derivative and a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, antipyretic and analgesic activities. Naproxen inhibits the activity of the enzymes cyclo-oxygenase-I and II, resulting in a decreased formation of precursors of prostaglandins and thromboxanes. The resulting decrease in prostaglandin synthesis is responsible for the therapeutic effects of naproxen. Naproxen also causes a decrease in the formation of thromboxane A_2 synthesis by thromboxane synthase thereby inhibiting platelet aggregation.

Pantoprazole chemically is 6- (difluoromethoxy)- 2- [(3,4- dimethoxypyridin – 2 – yl) methylsulfinyl] -1*H*- benzimidazole Fig. 1 Pantoprazole is a substituted Benzimidazole and proton pump inhibitor with antacid activity. Pantoprazole is a lipophilic weak base that crosses the parietal cell membrane and enters the acidic parietal cell canaliculus where it becomes protonated producing the active metabolite sulphonamide, which forms an irreversible covalent bond with two sites of the H+/K+-ATPase enzyme located on the gastric parietal cell, thereby inhibiting both basal and stimulated gastric acid production.

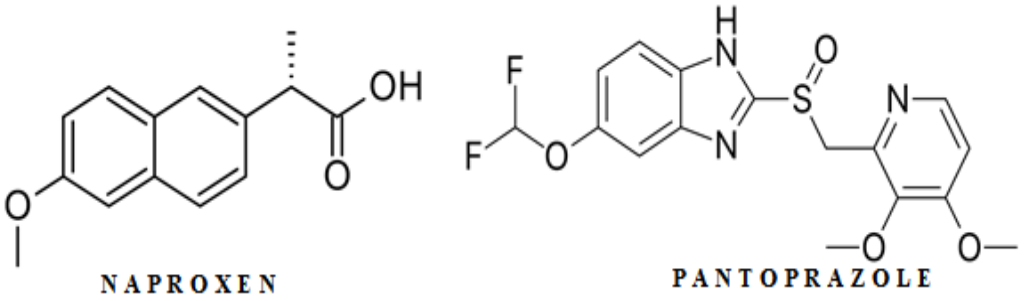

Fig. 1: Structure of Naproxen and Pantoprazole

Several analytical methods have also been reported for the determination of naproxen in pharmaceutical preparations including UV-visible spectrophotomery [4-6] spectrofluorimetry [7], phosphorimetry [8], voltammetry [9], high-performance liquid chromatography [10-12], capillary electrophoresis [13-14], coulometry [15] and oscilometric titration [16]. In our studies no method has been reported for simultaneous estimation of these drugs in combination using QbD based 3^3 factorial designing.

Chemicals and Reagents

Reference standards of Naproxen hydrochloride was obtained as gift sample from Dr. Reddy's Laboratories, Hyderabad, India while Pantoprazole was obtained as generous gift from Micro Labs Ltd., Bangalore, India. Pharmaceutical formulation was purchased from local market (Brand: Arthopan tablet labelled claim Naproxen 500 and Pantoprazole 20 mg make Crescent Therapeutics Ltd.). The HPLC grade solvents used were of E-Merck (India) Ltd., Mumbai. HPLC grade Methanol and Ortho Phosphoric Acid (Merck, Mumbai, India) were used in the analysis. HPLC grade water was prepared using Millipore purification system.

Instruments

The analysis of the drug was carried out on Agilent Tech. Gradient System with Auto injector, UV (DAD) & Gradient Detector. Equipped with Reverse Phase (Agilent) C_{18} column (4.6mm x 100mm; 2.5µm), a 20µl injection loop and UV730D Absorbance detector and running chemstation 10.1 software.

RP-HPLC Optimised Chromatographic Condition using QbD

Column C_{18} (100 mm× 4.6mm); particle size packing 5µm; detection wavelength 231 nm; flow rate 0.7 ml/min; temperature 26^0C ambient; sample size 20 µl; mobile phase methanol: water (OPA 0.1% PH 3) (75:25); run time 15 min. The retention time for Naproxen and Pantoprazole were found at 2.9333 min and 6.9667 min respectively Fig. 2.

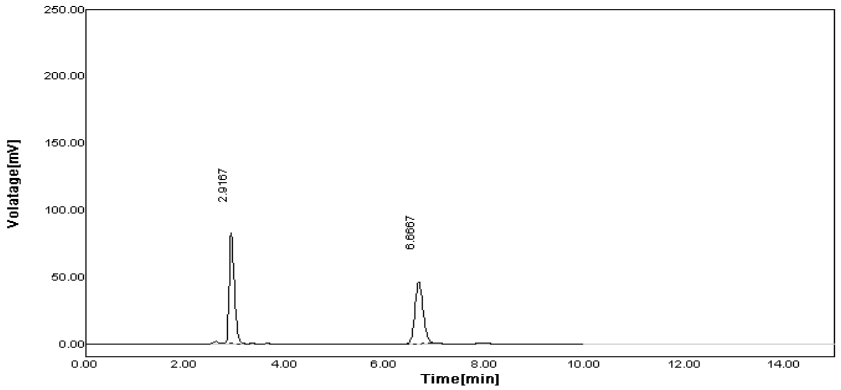

Fig. 2: Chromatogram of standard NPX and PPZ at 239 nm

Preparation of standard solution

All solutions were prepared on a weight basis and solution concentrations were also measured on weight basis to avoid the use of an internal standard. Pharmaceutical formulation which is available in the market is with the proportion of 250:2 thus the concentration was made by adding standard drug to obtain the proportion as 2.5:1.

Stock preparations

Standard stock solution was prepared by dissolving 10 mg NPX and PPZ in 10 ml clean dry volumetric flask and dilution was upto the mark with Methanol to obtain final concentration of NPX (1000 µg/ml) and PPZ (1000 µg/ml). All the stock solutions were filtered through 0.45 µm membrane filter.

Detection of λ_{max}

The sample solution has been prepared and scanned in the UV region of 200-400 nm and the spectrum showed the maximum absorbance at 239 nm Fig. 3.

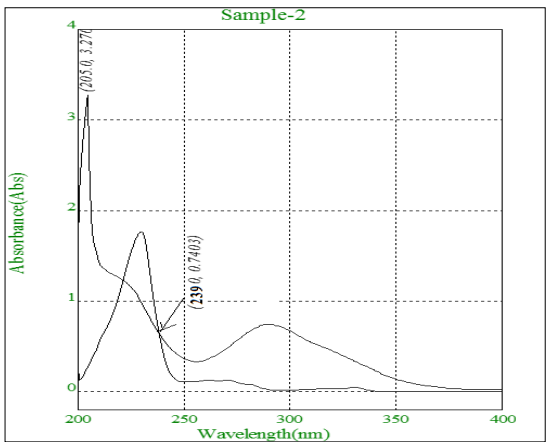

Fig. 3: Simultaneous estimation at 239 nm on UV spectrophotometer

QbD approach to analysis

The application of QbD in HPLC method development commences with establishing analytical objectives based on sound science to ensure consistent method performance characteristics are achieved. The use of QbD for an analytical method commences with defining the target analytical profile in which the pre-defined objectives for method performance must be appropriately validated and documented. Thus the objective of this work was to perform experimental design by using Design Expert Software leading to develop simple, rapid and sensitive method by QbD approach and validated as per ICH Guidelines (Q2R1) for Naproxen and Pantoprazole and its stability indicating method by RP-HPLC. Further statistical data analysis to be done along with numerical and graphical optimization to develop Analytical Design Space (ADS).

Method Validation

Calibration Curve

A calibration curve was constructed succeeding replicate (n=6) analysis of five standards of 10, 20, 30, 40 and 50 µg/ml of NPX and 4, 8, 12, 16 and 20 µg/ml PPZ. The peak height ratio of drugs was calculated and plotted AUC versus concentration after which least squares linear regression analysis of data was undertaken to establish the equation for the best fit line and the correlation coefficient (R^2) to authorise linearity. Samples were injected and peaks were recorded at 239 nm and the graph plotted as concentration of drug verses peak area as shown in Table 1.

Table 1: Linearity study

NPX			PPZ		
Conc. [µg/ml]	Mean peak area ± SD [n=5]	%RSD	Conc. [µg/ml]	Mean peak area ± SD [n=5]	%RSD
10	208.8±3.70	1.77	4	173.6±3.05	1.76
20	412.2±6.87	1.67	8	311.4±5.98	1.92
30	619.4±5.81	0.94	12	453.0±7.28	1.61
40	823.2±7.12	0.86	16	571.6±4.83	0.84
50	1055.4±9.34	0.89	20	729.6±5.90	0.81

Precision

Intra-day (repeatability) precision was established following analysis of replicate samples (n=6) at three concentrations indicative of low, medium and high levels within the linear range *viz.*, 20, 30 and 40 µg/ml of NPX and 8, 12 and 16 µg/ml PPZ. Analysis was performed over a short period of time on the same day. Inter-day precision or reproducibility was assessed at low, medium and high concentration on three consecutive days and the percent relative standard deviation (% RSD) was used to assess intra- and inter-day precision. An upper limit of 2% was used to confirm precision in our laboratory. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Table 2-3 describes the Intraday, Interday and Repeatability of method.

Table 2: Results of precision studies (Intra-day and Inter-day)

Drug	Conc. [µg/ml]	Intraday Amount Found [µg/ml]		Inter day Amount Found [µg/ml]	
		Mean± S.D.	% RSD [n= 3]	Mean ±S.D.	% RSD [n= 3]
NPX	20	29.87 ±4.16	0.34	29.51 ± 8.50	0.69
	30	44.37±10.21	0.55	44.77±7.64	0.41
	40	59.50±6.66	0.27	59.50±9.45	0.38
PPZ	8	3.77±2.00	0.29	4.13±2.00	0.28
	12	6.25±3.06	0.27	6.09±5.57	0.50
	16	8.39±5.51	0.35	7.65±5.03	0.34

Table 3: Results of repeatability study

Drug	Concentration [µg/ml] [n=6]	Peak Area	Mean[µg/ml] ± SD	% RSD
NPX	30	622.833	45.15 ± 0.94	1.26
PPZ	12	377.677	6.25 ± 0.255	1.677

Accuracy

Recovery studies were performed to validate the accuracy of developed method. To pre-analysed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed**.** Statistical validation of recovery studies shown in Table 4.

Table 4: Results of recovery studies

Drug	Initial amount [µg/ml]	Amount added [µg/ml]	Amt. recovered ± S.D. [µg/ml, n =3]	% Recovery	% RSD
NPX	30	0	45.29 ±0.67	100.39	0.89
	30	24	80.89 ±0.89	99.81	1.49
	30	30	89.69 ±1.09	99.58	1.45
	30	36	144.49 ±1.28	100.55	1.42
PPZ	12	0	6.15 ±0.27	100.99	1.83
	12	9.6	10.87 ±0.20	100.57	1.67
	12	12	12.08 ±0.24	100.51	1.58
	12	14.4	12.84 ±0.18	99.11	1.02

Limits of detection (LOD) and quantitation (LOQ)

Several approaches for the calculation of the LOD and LOQ of a method have been suggested in different guidelines and include visual evaluation, use of signal-to-noise (S/N) ratio, calculations based on standard deviation of a response and the slope of the calibration curve. By convention method, the LOD is estimated as one third of the LOQ. A series of samples of 10, 12, 14, 16, 18 and 20 µg/ml of NPX and 4, 5, 6, 7 and 8 µg/ml of PPZ were prepared and analysed using the optimized RP-HPLC method and the peak height ratio calculated. The LOQ was determined by establishing the lowest concentration of drugs that resulted in a % RSD value for precision of <2 %.

Specificity

The specificity of an analytical method is defined as the ability of a method to ensure that the peak(s) of interest elute as distinct responses in the presence of excipients, impurities or degradation compounds.

Robustness

To evaluate robustness few parameters were deliberately varied. The parameters include variation of flow rate, percentage of methanol as described in Table 5.

Table 5: Robustness evaluation of the HPLC method

Chromatographic conditions	NPX			PPZ		
	Tailing (T')	Capacity Factor (K')	Theoretical Plate (N)	Tailing (T')	Capacity Factor (K')	Theoretical Plate (N)
A: Mobile phase pH						
2.8	1.26	1.23	2683.9	1.28	0.99	7591.4
3.0	1.22	1.27	2683.5	1.23	1.09	7632.5
3.2	1.21	1.33	2625.5	1.25	1.15	7414.7
Mean ±SD	1.23±0.02	1.27±0.05	2687.63±36.80	1.25±0.02	1.07±0.02	7546.2±115.7
B: Flow rate (ml/min.)						
0.5 ml	1.23	0.98	2723.8	1.26	0.76	7587.3
0.7 ml	1.16	1.08	2818.9	1.29	1.10	7668.8
1.0 ml	1.15	1.09	2768.7	1.22	0.88	7423.5
Mean ±SD	1.18±0.04	1.05±0.06	2770.47±47.50	1.25±0.03	0.91±0.17	7593.2±75.82
C: Percentage methanol in mobile phase (v/v)						
65	1.09	1.22	2646.2	1.18	0.87	7623.8
75	1.06	1.13	2687.4	0.94	0.95	7667.3
85	1.19	1.18	2638.3	1.23	0.87	7433.2
Mean ±SD	1.11±0.06	1.17±0.04	2657.3±26.36	1.11±0.15	0.89±0.04	7574±124.51

Study of system suitability parameters

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution as shown in Table 6.

Table 6: System suitability test

NPX		PPZ	
System suitability parameters	Proposed method	System suitability parameters	Proposed method
Retention time (Rt)	2.9333	Retention time (Rt)	6.9167
Capacity factor (K')	1.18	Capacity factor (K')	0.98

Theoretical plate (N)	2838.7	Theoretical plate (N)	7465.8
Tailing factor (T)	1.16	Tailing factor (T)	0.95

Forced degradation studies

Forced degradation study was performed to evaluate the stability of the developed method using the stress conditions like exposure of sample solution to acid, base, Hydrogen peroxides (H_2O_2) and Neutral. Investigation were done for the degradation products in different conditions and are shown in Table 7.

Procedure for Naproxen and Pantoprazole degradation

Acid hydrolysis

The acid hydrolysis performed using 0.1N HCl at 70 °C for 1st hr and 2nd hr for both Pantoprazole and Naproxen indicated degradation. The major degradation products for Pantoprazole and Naproxen were observed at relative retention time (RRT) for 1st and 2nd Hours.

Alkaline hydrolysis

The alkaline hydrolysis condition was performed using 0.1N NaOH at 70 °C for 1st hr and 2nd hr both Pantoprazole and Naproxen. The major degradation products for Pantoprazole and Naproxen were observed at relative retention time (RRT) for 1st and 2nd Hours

Oxidation

In the oxidation condition with 3% H_2O_2 for 1st hr and 2nd hr both Pantoprazole and Naproxen show oxidative stress degradation peak in the chromatogram.

Neutral

There was no major degradation observed for both Pantoprazole and Naproxen and hence they were not sensitive to light at 70 °C for 1st hr and 2nd hr.

Table 7: Forced degradation

Sample Exposure condition	Total Number of products with their Rt	NPX		PPZ	
		Degradation remained (30 µg/ml)	Recovery (%)	Degradation remained (12 µg/ml)	Recovery (%)
Acidic, 1N, 1 Hr	5 (2.95, 4.80,6.05,7.08,7.65)	136.224	90.81	28.25	94.18
Basic, 1N, 1 Hr	6 (2.61, 2.80, 2.95, 3.38, 4.51, 7.20)	122.22	81.48	13.28	44.29
Per oxide, 30%, 1 Hr	4 (2.63, 2.83, 4.76, 7.03)	128.50	85.67	20.92	69.73
Heat, 50°C, 1 Hr	3 (2.61,2.81,6.766)	136.58	91.05	22.20	74.01

Application of analytical method

To determine the content of NPX and PPZ in marketed tablets (label claim 500 mg of Naproxen and 20 mg Pantoprazole), 20 tablets powder weighed as 5.96 gm and average weight of powder was calculated. Tablets were triturated and powder equivalent to weighed and drug was extracted from the tablet powder with 10 ml Methanol. To ensure complete extraction it was sonicated for 15 min. 0.1 ml of supernatant was then diluted up to 10 mL with mobile phase. The resulting solution was injected in HPLC and drug peak area was noted**.**

Results and Discussion

Such analytical methods are, in fact, an indicator of a quality product and the robustness of that product for the duration on the lifecycle of that product. The main goal of any HPLC method is to separate and quantitate analyte(s) of interest from any impurity and/or excipients. Initially it is important to establish the critical quality attributes (CQA) of a system that may impact the quality of the analytical method. Development of Analytical RP-HPLC Method with Design Space and Control Strategy determination by optimization study all the computations for the current optimization study and statistical analysis were performed using Design Expert® software (Design Expert trial version). State-Ease Inc., Minneapolis, MN, USA).

Application of design of experiments for method optimization Design of experiments (DOE-1)

Thus, 3 randomized response surface designs with a full fraction design were used with 17 trial runs to study the impact of three factors on the three key response variables. In this design 3 factors were evaluated, each at 3 levels, and experimental trials were performed at all 3 possible combinations. The mobile phase composition (X1), Wavelength (X2) and flow rate (X3), were selected as independent variables and retention time (RT) and Resolution were selected as dependent variables. The resulting data were fitted into Design Expert 10 Software and analyzed statistically using analysis of variance (ANOVA) and F-Test. Figure 3 indicates the normal plot of residuals for retention time with other chromatographic parameters. The data were also subjected to 3-D response surface methodology to determine the influence of flow rate, Wavelength and mobile phase composition on dependent variables as shown in Figure 4. The probable trial runs using 3^3 full fraction designs are as shown in Table 4. Further ANOVA and F-test with variables are shown in Table 8-12. More over degradation peaks of API were shown in Figure 5-8 from acidic, alkaline, peroxide and Heat.

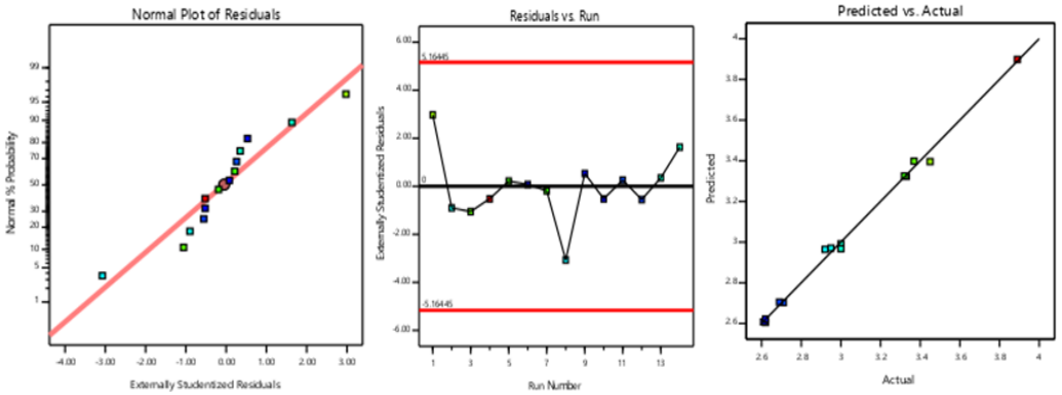

Fig. 3A: Normal plot of residuals for retention time and plot of predicted vs. actual data for retention time of cp

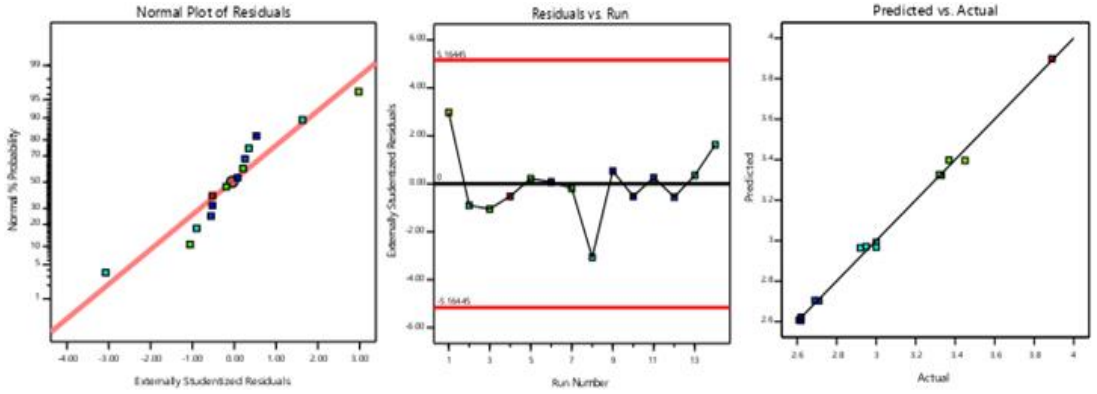

Fig. 3B: Normal plot of residuals for retention time and plot of predicted vs. actual data for retention time by the value of 2.61 to 3.89

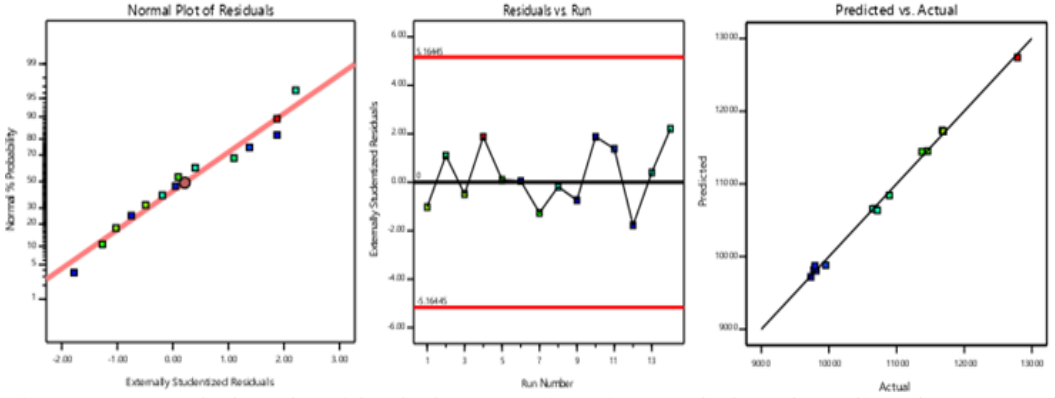

Fig. 3C: Normal plot of residuals for retention time and plot of predicted vs. actual data for retention time by the value of 9733 to 12789

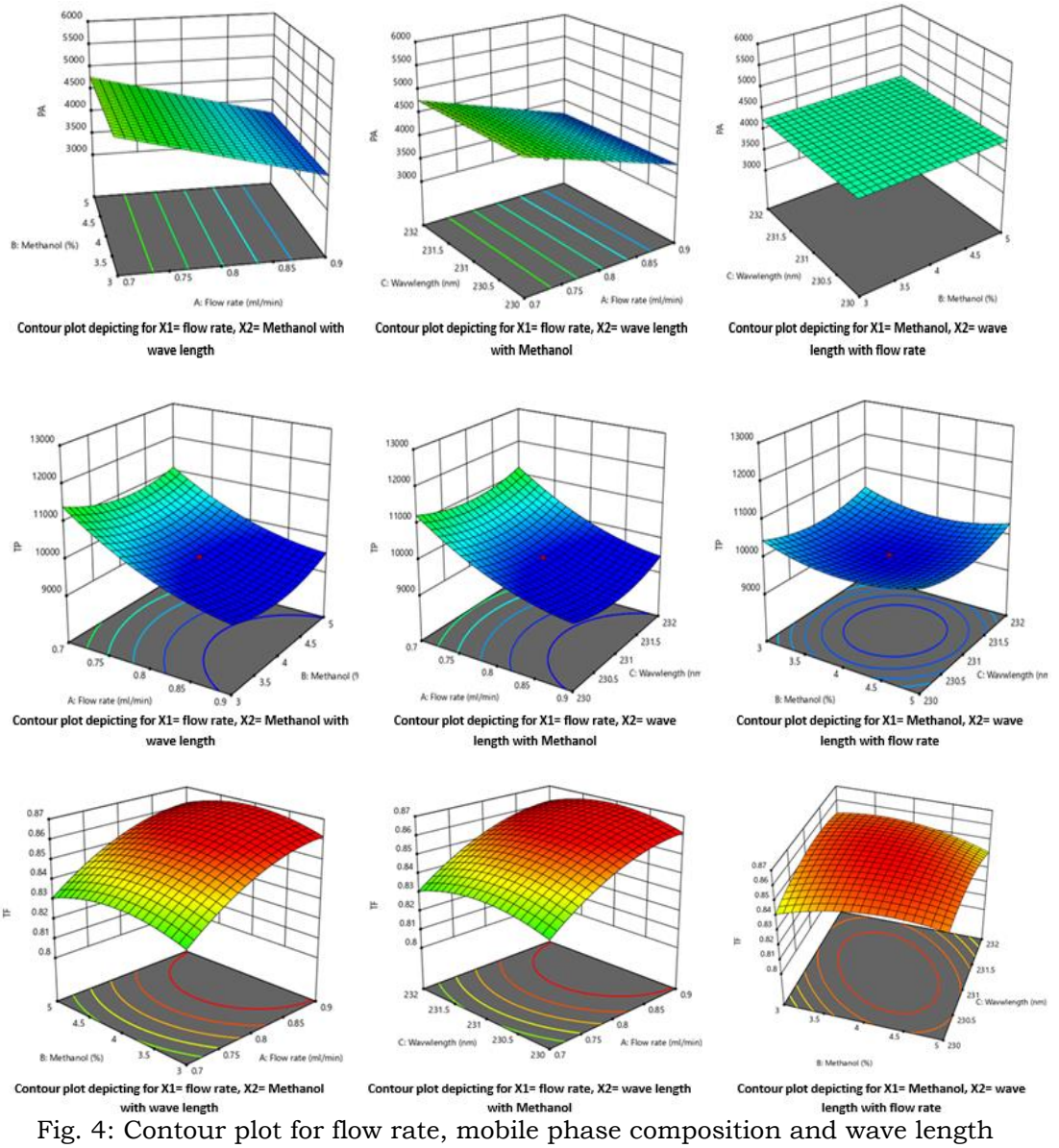

Fig. 4: Contour plot for flow rate, mobile phase composition and wave length

Table 8: Probable trial runs using 3^3 full fraction designs

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
		A:Flow rate	B:Methanol	C:Wave length	RT	PA	TP	TF
		ml/min	%	nm				
1	1	0.7	3	230	3.45	4850.37	11675	0.82
11	2	0.8	2.3	231	2.95	4019.71	10892	0.84
5	3	0.7	3	232	3.369	4521.28	11693	0.83
9	4	0.6	4	231	3.89	5516.24	12789	0.8

3	5	0.7	5	230	3.33	4896.5	11458	0.83
6	6	0.9	3	232	2.61	3555.04	9810	0.86
7	7	0.7	5	232	3.32	4665.06	11373	0.82
13	8	0.8	4	229.3	2.92	4373.36	10645	0.84
2	9	0.9	3	230	2.62	3755.37	9777	0.85
10	10	0.8	4	231	2.62	3707.75	9733	0.86
4	11	0.9	5	230	2.71	4018.88	9950	0.86
8	12	0.9	5	232	2.69	3785.56	9793	0.85
12	13	0.8	5.7	231	3	4326.7	10679	0.84
14	14	0.8	4	232.7	3	4484.22	10716	0.84

Table 9: Anova for reduced quadratic model (response 1: rt)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.95	7	0.2783	211.69	< 0.0001	significant
A-Flow rate	1.10	1	1.10	833.33	< 0.0001	
B-Methanol	0.0005	1	0.0005	0.4083	0.5464	
C-Wavelength	0.0000	1	0.0000	0.0124	0.9149	
AB	0.0144	1	0.0144	10.93	0.0163	
A^2	0.1479	1	0.1479	112.53	< 0.0001	
B^2	0.0882	1	0.0882	67.09	0.0002	
C^2	0.0810	1	0.0810	61.60	0.0002	
Residual	0.0079	6	0.0013			
Cor Total	1.96	13				

Table 10: Anova for reduced linear model (response 2: pa)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.294E+06	1	3.294E+06	57.63	< 0.0001	significant
A-Flow rate	3.294E+06	1	3.294E+06	57.63	< 0.0001	
Residual	6.858E+05	12	57151.71			
Cor Total	3.979E+06	13				

Table 11: Anova for reduced quadratic model **(**response 3: tp)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.120E+07	7	1.600E+06	288.01	< 0.0001	significant
A-Flow rate	6.682E+06	1	6.682E+06	1202.92	< 0.0001	
B-Methanol	40072.40	1	40072.40	7.21	0.0363	
C-Wavwlength	358.64	1	358.64	0.0646	0.8079	
AB	60031.13	1	60031.13	10.81	0.0167	
A^2	7.371E+05	1	7.371E+05	132.68	< 0.0001	
B^2	7.252E+05	1	7.252E+05	130.54	< 0.0001	
C^2	5.848E+05	1	5.848E+05	105.27	< 0.0001	
Residual	33330.78	6	5555.13			
Cor Total	1.123E+07	13				

Table 12: Anova for reduced quadratic model **(**response 4: tf)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0040	7	0.0006	646.40	< 0.0001	Significant
A-Flow rate	0.0020	1	0.0020	2334.61	< 0.0001	
B-Methanol	0.0000	1	0.0000	0.0000	1.0000	
C-Wavelength	0.0000	1	0.0000	0.0000	1.0000	
BC	0.0002	1	0.0002	228.17	< 0.0001	
A^2	0.0004	1	0.0004	427.66	< 0.0001	
B^2	0.0003	1	0.0003	298.35	< 0.0001	
C^2	0.0003	1	0.0003	298.35	< 0.0001	
Residual	5.259E-06	6	8.765E-07			
Cor Total	0.0040	13				

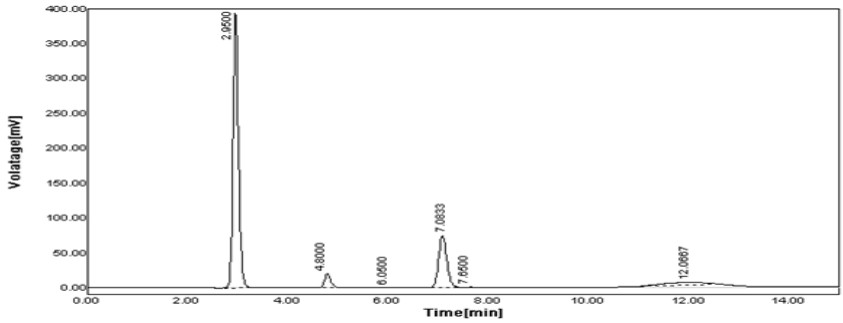

Fig. 5: Acidic degradation

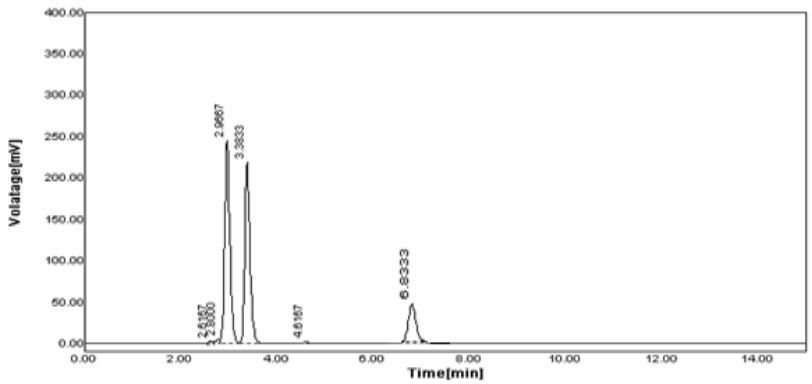

Fig. 6: Alkaline degradation

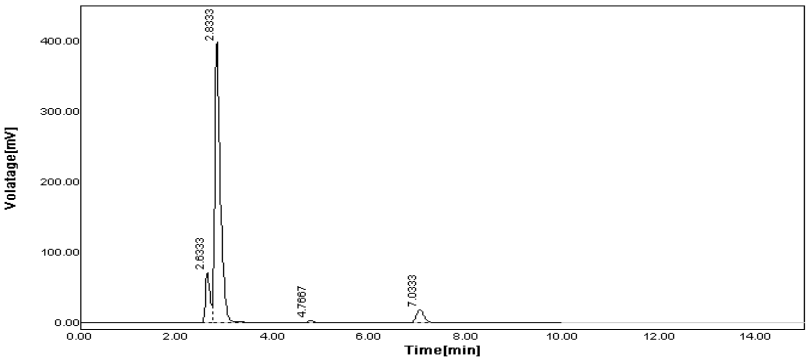

Fig. 7: Per oxide degradation

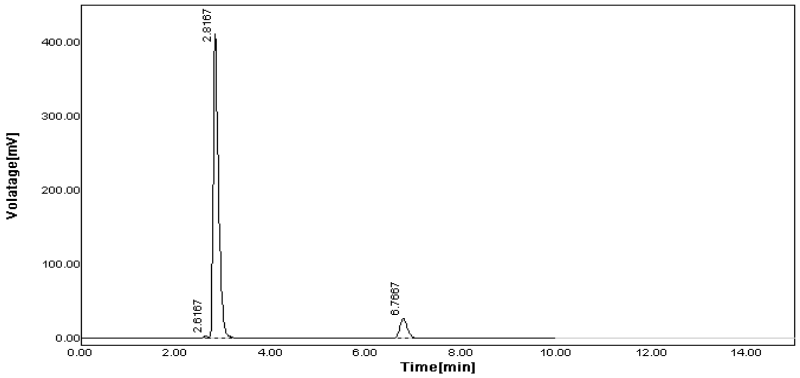

Fig. 8: Heat degradation

Conclusion

A simple, rapid, reliable, robust and optimized reversed phase high performance liquid chromatographic method for estimation of Naproxen and Pantoprazole was successfully developed and validated as per International Conference on Harmonization guidelines. Percentage of mobile phase, flow rate and wave length were optimised by using QbD approach *i.e.* 3^3 factorial design. There are no interfering peaks underperformed degradation conditions. Therefore, a sensitive, accurate and stability indicating method was developed with high degree of practical utility.

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Conflicts of interest

The authors declare no conflicts of interest.

Abbreviation used

HPLC: High performance liquid chromatography; UV: Ultraviolet; ICH: International Conference on Harmonization; LOQ: Limit of quantitation; LOD: Limit of detection; RSD: Relative standard deviation; RT: Retention time; OPA: Orthophosphoric acid; NPX: Naproxen; PPZ: Pantoprazole; FDA: Food and Drug Administration; SD: Standard deviation.

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