IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
September 2015 Vol.:4, Issue:2

Quality by Design Approach to Analytical Method Development for Simultaneous Estimation of Hesperidin Methyl Chalcone, Hesperidin and Ascorbic Acid in Their Combined Dosage Form by RP-HPLC Method

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Bhoomi Patel*, Karan Mittal, Dharmendra Damor, Rajshree Mashru

Quality Assurance Laboratory, Centre of Relevance and Excellence in Novel Drug Delivery system, Pharmacy Department, Shree G.H.Patel Pharmacy building, Donor's Plaza, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara-390002, Gujarat, India.

Submission: 22 August 2015
Accepted: 29 August 2015
Published: 25 September 2015

Keywords: Quality by Design, HPLC, Hesperidin, Ascorbic acid, Design Expert

ABSTRACT

A simple, specific, precise method for simultaneous estimation of Hesperidin methyl chalcone, Hesperidin and Ascorbic acid has been developed. Separation was achieved on Kromasil C18 column (250 x 4.6 mm, 5 µm) using 10 mM phosphate buffer pH 4.2 adjusted using dilute orthophosphoric acid : Methanol : Acetonitrile (50:40:10% v/v/v) with 0.05% TEA with flow rate of 0.9 mL/min at ambient temperature. A Quality by Design (QbD) was employed for optimization of the method. 3 variables pH of buffer, Initial % Acetonitrile and flow rate had been varied and a model highlighting allowed design space was generated. By employing DoE approach, a better chromatographic method was obtained in which some of the initially merged peaks in the chromatogram were resolved successfully and good symmetric peaks of AA and HMC were also obtained. HMC, HP and AA were eluted at retention time of about 6.047 min, 4.658 min and 2.561 min respectively. The developed method was successfully validated according to ICH Q2 (R1) guidelines. Linearity was observed for HMC, HP and AA in range of 5-25 µg/ml, 10-50 µg/ml and 25-75 µg/ml respectively with r2 values of 0.9951, 0.9991 and 0.9989 respectively. For intraday and interday precision %RSD for all drugs were found to be less than 2 indicating that the method was precise while recovery of HMC, HP and AA was found to be in range of 96-102%. The sample recovery was in good agreement with the respective label claim, which suggested non-interference of formulation additives in its estimation. Hence, the developed RP-HPLC method could be successfully applied for estimation of HMC, HP and AA in their combined dosage form.

1. INTRODUCTION

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[1-2].

Implementation of analytical QbD in pharmaceutical quality system is described in Table 1.

Hesperidin (HP; Fig. 1) and Hesperidin Methyl Chalcone (HMC; Fig. 2) are flavone glycoside found in peels and pulp of citrus fruits like oranges, lemon and grapefruit. Hesperidin is a aglycone part that is bound to the disaccharides rutinose. These bioflavonoids function synergistically with vitamin C (Ascorbic acid, AA; Fig. 3) in regard to maintain healthy capillaries, to help to form collagen in connective tissue, to help to heal wounds, and to support a healthy immune system[3-4].

Tablets (Peridin- C) are used in hot flashes associated with menopause and to improve the capillary strength.

Literature survey suggests that methods have been reported for estimation of HMC by HPLC[5], estimation of HP in human plasma by flourimetry[6], HPTLC[7], estimation of HP in combination with diosmin by spectroscopy[8] and HPLC[9], Stability indicating RP-HPLC method for HP and diosmin in combination[10], estimation of HP and naringin by HPLC[11], simultaneous estimation of HP, diosmin and eriocitrin by HPLC[12], Fast HPLC method for Rutin, truxerutin, diosmin and HP in food supplements[13]. Estimation of AA by HPLC[14], estimation of AA and calcium pantothenate by RP-HPLC[15], estimation of Ascorbic Acid and Gallic Acid in *Phyllanthus emblica* by HPLC[16], spectroscopic and RP-HPLC methods for AA in fruit juice and human plasma[17], spectroscopic estimation of AA with rutin[18].

The present work aimed to develop accurate, precise RP-HPLC method for simultaneous estimation of HP, HMC and AA in Quality by Design framework.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

HP and HMC standard were purchased from Sigma Aldrich. Standard AA was purchased from RFCL limited. Peridin C tablets were purchased online from www.amazon.com. Double distilled water (Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.2 µm pore size and 47 mm diameter (Pall Lifesciences, Mumbai, India). Acetonitrile and methanol HPLC grade were purchased from Spectrochem pvt. ltd, Mumbai. Potassium dihydrogen phosphate was purchased from Loba Chemicals Pvt. Ltd. (Mumbai, India).

2.2 Instrumentation and chromatographic condition

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) LC system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV detector and Rheodyne 7725 injector with fixed loop of 20 µl. Isocratic condition with mobile phase of Dihydrogen phosphate buffer pH 4.2 (10 mM): Methanol: Acetonitrile (50:40:10% v/v/v) with 0.05% TEA and 0.9 ml/min flow rate was used for analysis. pH of buffer was adjusted with orthophosphoric acid (OPA). Dihydrogen orthophosphate buffer was then filtered using 0.45 µm. Mobile phase was premixed and degassed using sonicator before use. Detection wavelength selected for estimation of all three drugs was 285 nm. Zero order overlain spectra of three drugs are shown in Fig. 4. Design expert© version 7 was used for design of experiment.

2.3 Preparation of standard solution

Standard solution of HP (100 µg/ml): 5 mg of HP was weighed accurately, transferred to 50 ml volumetric flak and dissolved in 10 ml methanol and diluted up to mark with 10 mM dihydrogen orthophosphate buffer (pH 4).

Same way 100 µg/ml HMC and AA were prepared by weighing standard HMC and AA respectively.

Mixture containing 30 µg/ml HMC, 30 µg/ml of HP and 5 µg/ml of AA was prepared from above standard solutions and was used for method development using QbD approach.

2.4 Method development by QbD approach

Various combination of phosphate buffer with methanol was tried to obtain well resolved chromatogram of HMC, HP and AA. But some additional peaks were found in standard drugs of HMC and HP as impurities. The impurities found in HMC overlapped with the peak of HP that leads to inadequate estimation of HP. So third component Acetonitrile was added in mobile phase to separate the HP peak from impurities. But this leads to increase the asymmetry of ascorbic acid (Fig. 5).

So, QbD approach was applied to get better resolution between impurities and standard drugs and optimization of such robust method to get good asymmetry of ascorbic acid too.

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.

2.4.1. Definition of method goals

Primary aim was to develop a more robust method and validation of developed method for simultaneous estimation of HMC, HP and AA in tablet dosage form. Quality by Design approach was applied 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 the method can meet the requirements of the ATP.

2.4.2 Risk assessment

In this stage of QbD approaches critical parameters that affect the overall quality of method were identified. Such as pH of buffer solution, ratio of acetonitrile (ACN) and flow rate were identified as critical parameters for simultaneous estimation of HMC, HP and AA in their combination.

2.4.3. Design of experiment

In design of experiment, three factors are considered: pH of buffer solution, %ACN and Flow rate were varied at three different levels. The critical responses studied were Asymmetry of AA, Resolution between impurity 1 and HP, resolution between impurity 2 and HP and asymmetry of HMC (Table 2).

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 our 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 (Fig. 6. For asymmetry of ascorbic acid, Fig. 7. For Resolution 1, Fig. 8. Resolution 2, Fig. 9. Asymmetry of HMC)

2.4.3.1 Optimization phase

Optimization criteria was selected for optimization of method that is shown in Table 3; several optimized solutions were generated by the software. From which 6 solutions were selected for check point analysis.

2.4.3.2 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 4. Desirability plot for selected working point is shown in Fig. 10.

2.4.4 Design space

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

2.4.5 Final optimized chromatographic method

- Column: Kromasil C18 (250 × 4.6 mm; 0.5 µm particle size)
- Mobile phase: Dihydrogen phosphate buffer pH 4.2 (10 mM): Methanol: Acetonitrile 50:40:10% v/v/v with 0.05% TEA

- Flow rate: 0.9 ml/min
- Temperature: Ambient
- Detection wavelength: 285 nm

2.5 Method validation [19]

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

2.5.1 Linearity

From standard solution of HMC, HP and AA HMC 5-25 µg/ml, HP 10-30 µg/ml and AA 15-75 µg/ml were prepared. Regression equation, Correlation coefficient, Slope and Intercept were calculated. Overlay chromatogram of HMC, HP and AA is shown in Fig. 13.

2.5.2 Precision

Intraday and inter day precision of developed methods was measured in terms of %RSD. All the methods were repeated 3 times in a day for intra-day and on 3 different days for inter-day precision. The average %RSD of intra-day and inter-day measurements for determination of all the drugs was found to be less than 2.

2.5.3 Accuracy

Accuracy of method was assessed by recovery study from formulation at three level of standard addition (50%, 100% and 150%) in triplicate. % recovery within 95-105% with low standard deviation justified the accuracy of developed method.

2.5.4 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

2.5.5 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.

2.5.6 System suitability parameters

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.

2.6 Applicability of developed method

20 Tablets were accurately weighed and crushed. Powder equivalent to 5 mg HMC, 15 mg HP and 20 mg AA 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 Phosphate buffer pH 4 and filtered through Whatman filter paper grade 1. Appropriate dilution was made and solution was subjected to analysis. % Assay obtained is shown in Table 9.

3. RESULTS AND DISCUSSION

3.1 Method development

Isocratic RP-HPLC method was developed for simultaneous estimation of HMC, HP and AA by applying QbD approach. Using this approach method was successfully optimized. Optimized chromatographic conditions were Column: Kromasil C18 (250 × 4.6 mm; 0.5 µm particle size; Mobile phase: Dihydrogen phosphate buffer pH 4.2 (10 mM): Methanol: Acetonitrile 50:40:10% v/v/v with 0.05% TEA with Flow rate of 0.9 ml/min.

3.2 Method validation

Method was successfully validated according to ICH Q2(R1) guidelines.

Beer's law was obeyed in range of 5-25 µg/ml for HMC, 10-50 µg/ml for HP, 15-75 µg/ml for AA. It showed 0.9951, 0.9991 and 0.9989 r^2 values for HMC, HP and AA respectively, 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 95-105% shows good accuracy of developed method. Developed method was also applied to tablet dosage form. %Assay obtained for all three drugs were 96.2%, 98.95% and 101.38% for HMC, HP and AA respectively. All validation parameters were shown in Table 5. Recovery study data are shown in Table 6. 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-7). System suitability parameters were also studied and reported in Table 8.

4. CONCLUSION

An innovative Quality by Design approach has been applied for development of RP-HPLC method for simultaneous estimation of HMC, HP and AA in their combined dosage form. All previously defined method goals were met. A design space – a volume in which the method is robust is defined and visualized. Also, the predicted asymmetry of AA and HMC, resolution 1 and resolution 2 values are in excellent agreement with experimental values. The method was fully validated in compliance with ICH guidelines and a robustness study was performed by varying three chromatographic parameters at three levels. So, RP-HPLC method was successfully developed and validated for simultaneous estimation of HMC, HP and AA in their combined dosage form.

REFERENCES

1. Syed Salman Lateef and Vinayak AK, Quality-by-Design Approach to Stability Indicating Method Development for Linagliptin Drug Product, ©Agilent Technologies Inc., 2014;5991-3834EN
2. Ramalingam Peraman, Kalva Bhadraya, and Yiragamreddy Padmanabha Reddy, Analytical Quality by Design: A Tool for Regulatory Flexibility and Robust Analytics, Int. jou. ana. chem. 2015;Article ID 868727.
3.www.acu-cell.com/bio.html
4.www.phytochemicals.info/phytochemicals/hesperidin.php
5. Castillo J., Benavente O., Borrego F., Delrio J., Analysis of commercial hesperidin methyl chalcone by high performance liquid chromatography, Journal of chromatography. 1991;555: 285-290.
6. Leposava A. Pavun, Jasmina M. Dimitric Marcovic, Predrage T. Durdevic, Milena D. Jelicik-Sankov, Daniela B. Dikanovic, Andrija R. Ciric and Dusan L. Malesev, Development and validation of a fluorometric method for the determination of hesperidin in human plasma and pharmaceutical forms, *J.* Serb. Chem. Soc. 2012; 77: 1625-1640.
7. Parwez Alam, Aftab Alam, Md. Khalid Anwer, and Saleh I Alqasoumi; Quantitative estimation of hesperidin by HPTLC in different varieties of citrus peels; Asian Pac J Trop Biomed. 2014; 4: 262–266.

8. Doddi Srilatha,Mahesh Nasare, Borra Nagasandhya, Valluri Prasad, and Prakash Diwan, Development and Validation of UV Spectrophotometric Method for Simultaneous Estimation of Hesperidin and Diosmin in the Pharmaceutical Dosage Form, ISRN Spectroscopy. 2013; Article ID 534830, 4 pages.
9. Garima Mishra*, Vikas Kumar Srivastava and Abhinav Tripathi, Analytical method development and validation for assay of Diosmin and Hesperidin in combined tablet dosage form by RP- HPLC, Int. J. of Pharm. & Life Sci. 2013; 4: 2834-2839.
10. Anwer M.K., Jamil S., Ansari M.J., Al-Shdefat R. and Abdel-Kader M.S., A new improved Stability-Indicating RP-HPLC method for Determination of Diosmin and hesperidin in combination, *Int.* Res. J. Biological Sci. 2014; 3: 41-46.
11. S. Gorinstein, D. Huang, H. Leontowicz, M. Leontowicz, K. Yamamoto, R. Soliva-Fortuny, O. Martin Belloso, A. L. Martinez Ayala, and S. Trakhtenberg, Determination of naringin and hesperidin in citrus fruits by High Performance Liquid Chromatography. The antioxidant potential of Citrus fruits, Acta chromatographica. 2006; 17.
12.Saeidi, Hadmohammadi MR, Peyrovi M, Iranshahi M, Barfi B, Babaei AB, Dust AM, HPLC determination of hesperidin, diosmin and eriocitrin in Iranian lime juice using polyamide as an adsorbent for solid phase extraction, J Pharm Biomed Anal. 2011; 56: 419-22.
13. Dalibor Satinsky & Katerina Jagerova & Lucie Havlikova & Petr Solich, A New and Fast HPLC Method for Determination of Rutin, Troxerutin, Diosmin and Hesperidin in Food Supplements Using Fused-Core Column Technology, Food Anal. Methods. 2013; 6: 1353–1360.
14. Snezana S Mitic, Danijela A Kostic, Danijela C Naskovic-Dokic, Milan N Mitic, Rapid and Reliable HPLC Method for the Determination of Vitamin C in Pharmaceutical Samples, Trop J Pharm Res. 2011; 10: 105.
15. Ramniwas Dudhwal , Jagadish P C, Krishnamurthy Bhat, Aruna Mane, Simultaneous Estimation of Ascorbic Acid and Calcium Pantothenate in Multivitamin and Multimineral Tablets by Reverse-Phase HPLC, Der Pharma Chemica. 2011; 3: 375-381.
16. Laxman Sawant*, Bala Prabhakar and Nancy Pandita, Quantitative HPLC Analysis of Ascorbic Acid and Gallic Acid in Phyllanthus Emblica, J Anal Bioanal Techniques. 2010; 1: 111.
17.Muhammad Saeed Arayne, Najma Sultana and Zakia Bibi, Rapid and Specific Spectrophotometric and RP-HPLC Methods for the determination of Ascorbic acid in Fruit juices and Human plasma; J. Chem.Soc.Pak. 2009; 31.
18. Kareem M. Younes*, Mai A. Basha and Maissa Y. Salem, Spectrophotometric and chromatographic methods for the simultaneous determination of rutin and ascorbic acid in their pharmaceutical formulation; Der Pharma Chemica. 2014; 4: 111-121.
19. International conference on the harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) Q2 (R1), Validation of analytical procedures: text and
methodology, International conference on Harmonization, Geneva, 2005.

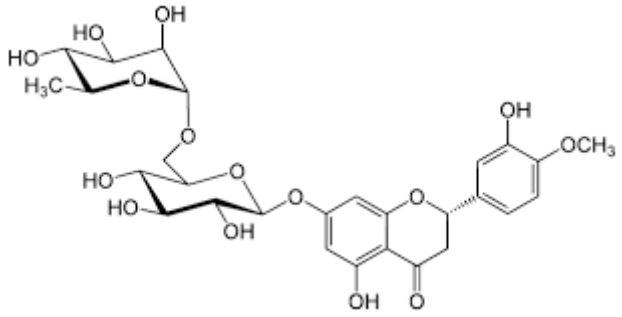

Fig. 1: Chemical structure of Hesperidin

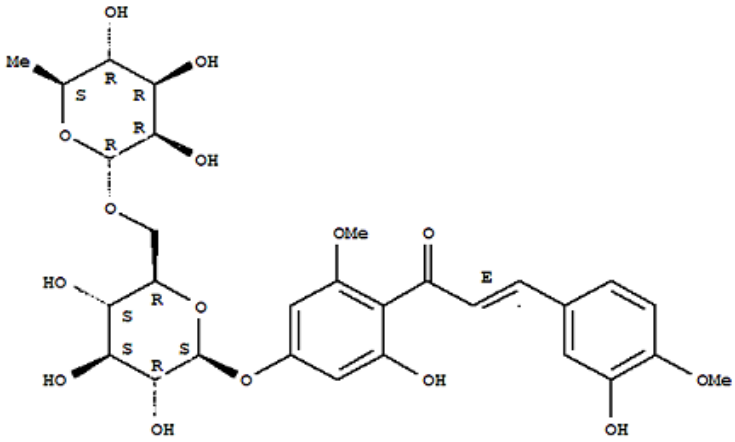

Fig. 2: Chemical structure of Hesperidin methyl chalcone

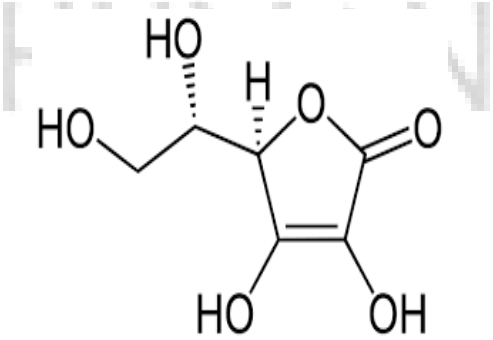

Fig. 3: Chemical structure of Ascorbic acid

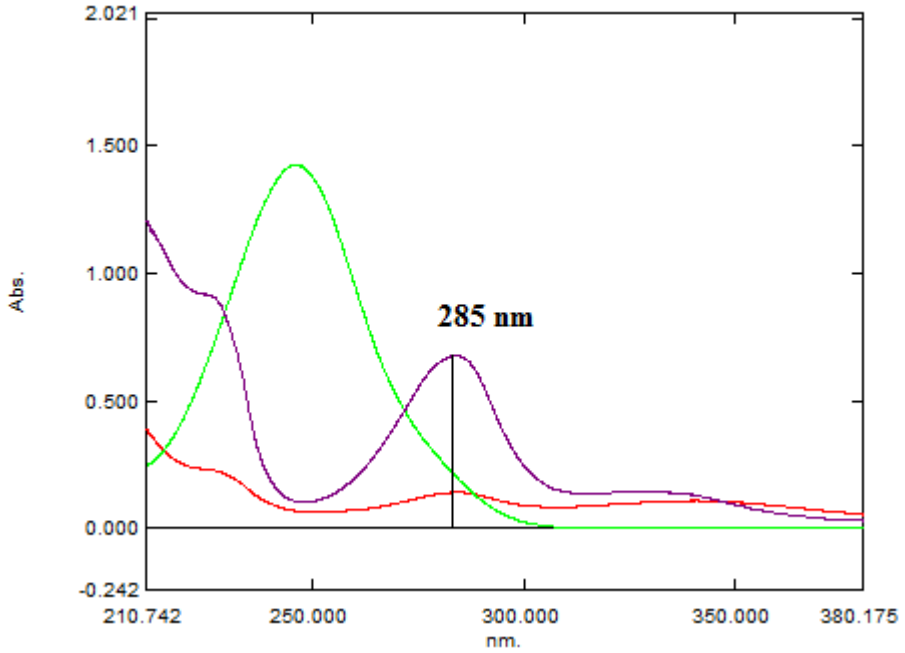

Fig. 4: Selection of detection wavelength

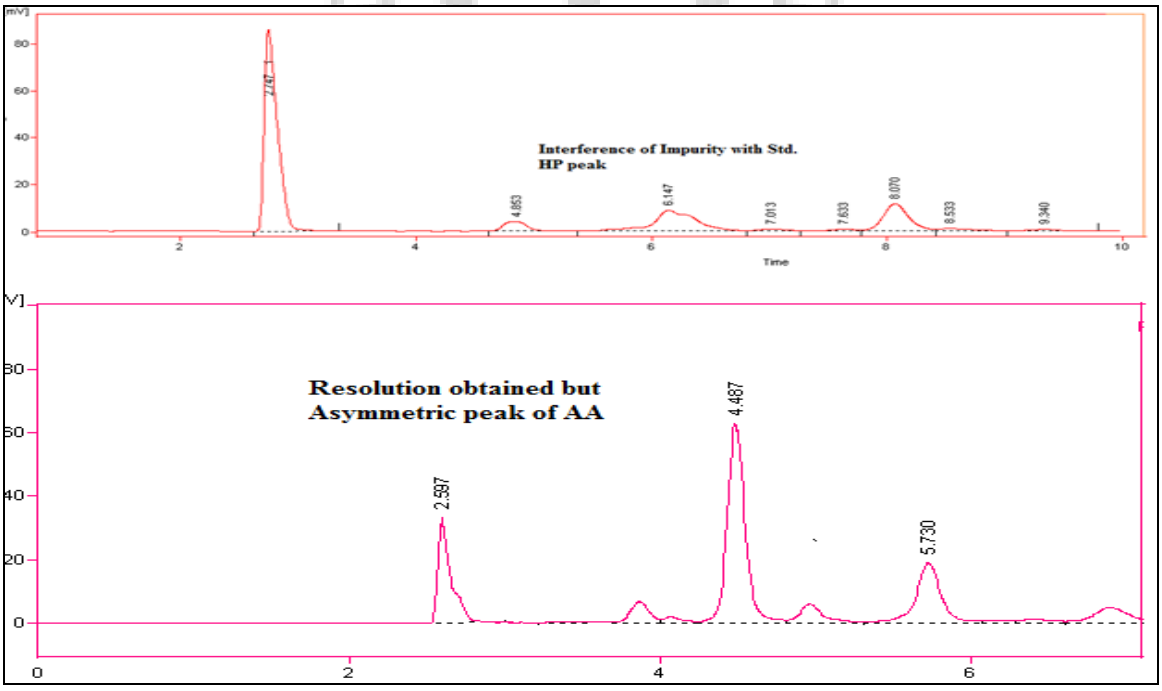

Fig. 5: Chromatogram of some preliminary trials

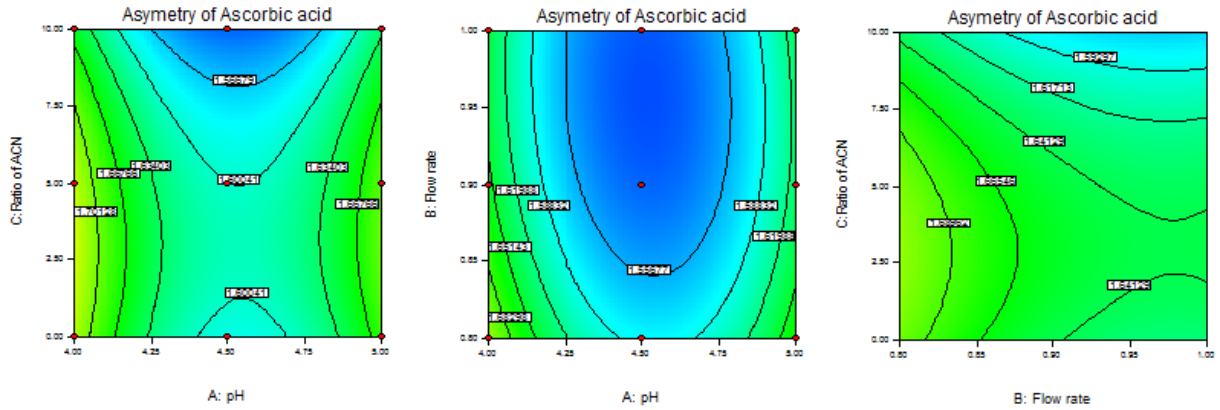

Fig. 6: Contour plots for Response 1 (Asymmetry of ascorbic acid)

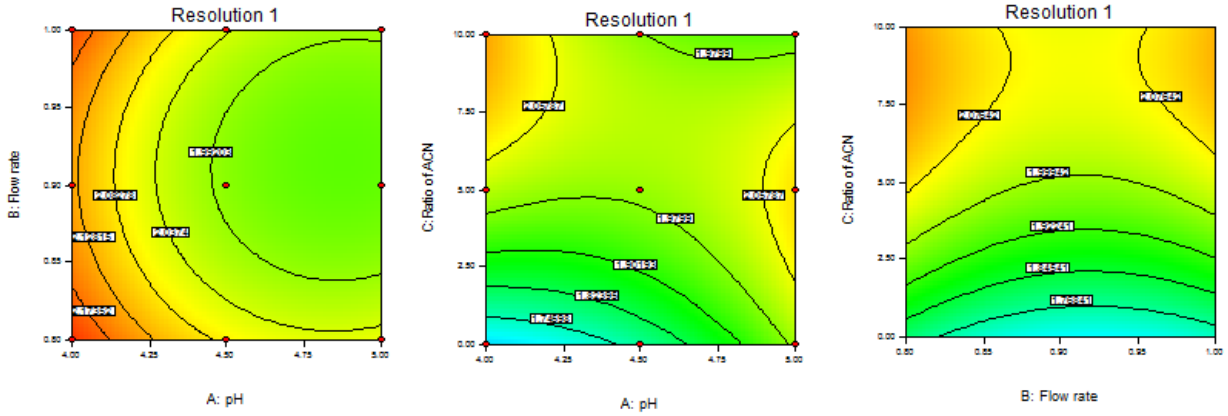

Fig. 7: Contour plots for Response 2 (Resolution between impurity 1 and HP)

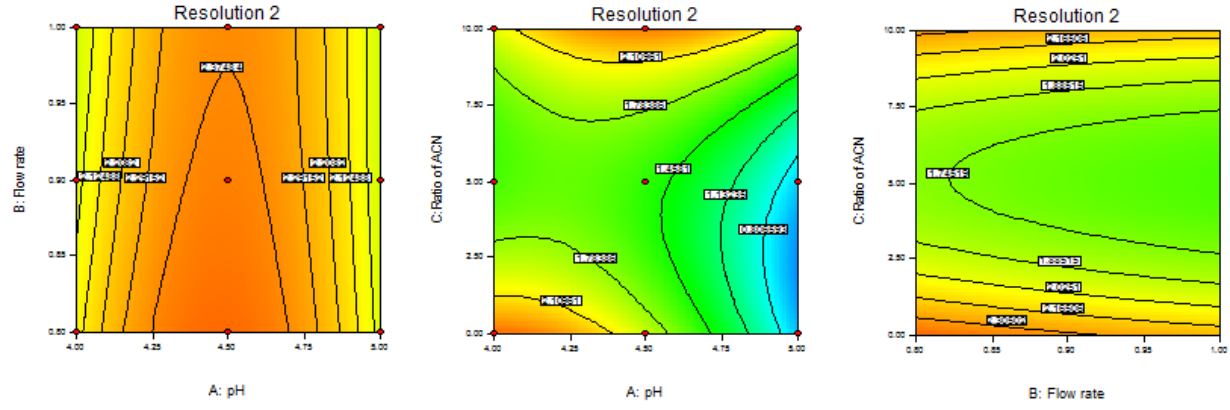

Fig. 8: Contour plots for Response 3 (Resolution between impurity 2 and HP)

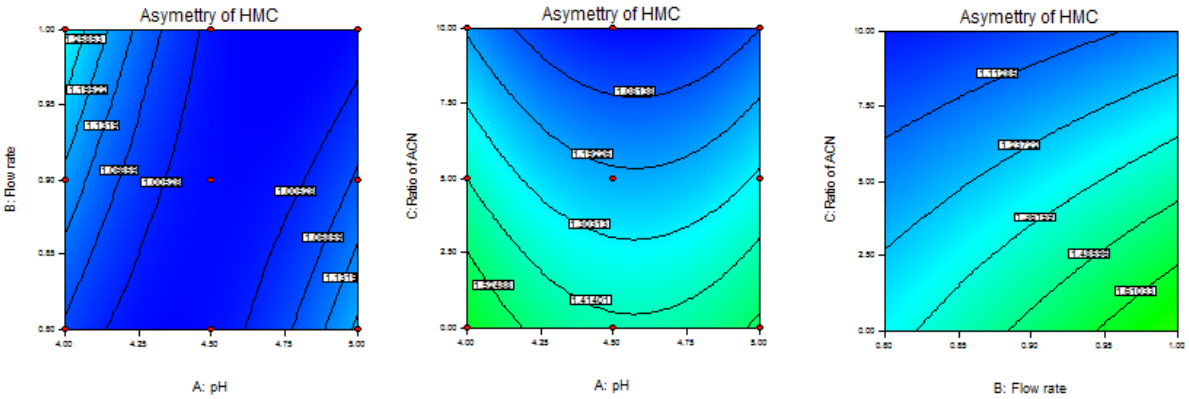

Fig. 9: Contour plots for Response 3 (Asymmetry of HMC)

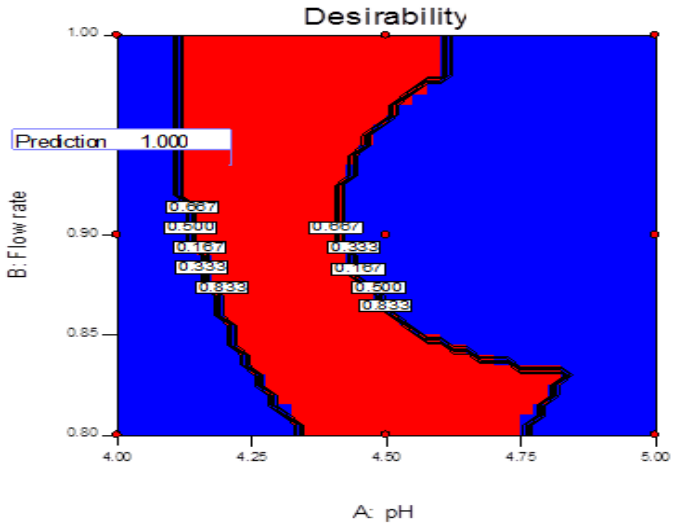

Fig. 10: Desirability graph for selected working point

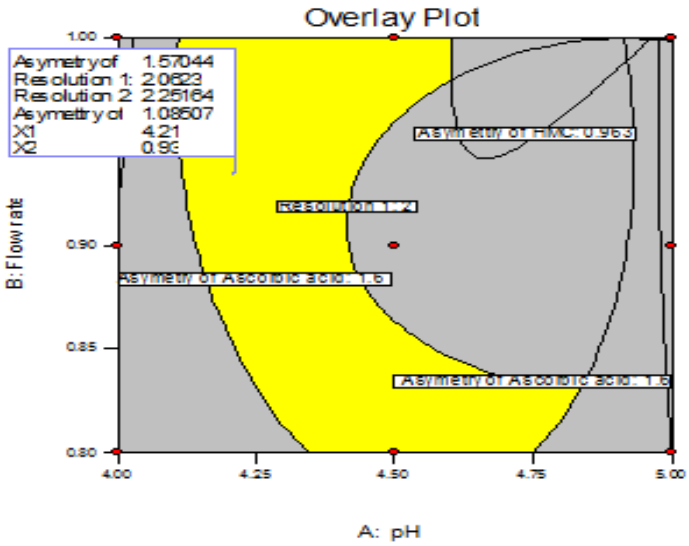

Fig. 11: Design space region for selected working point

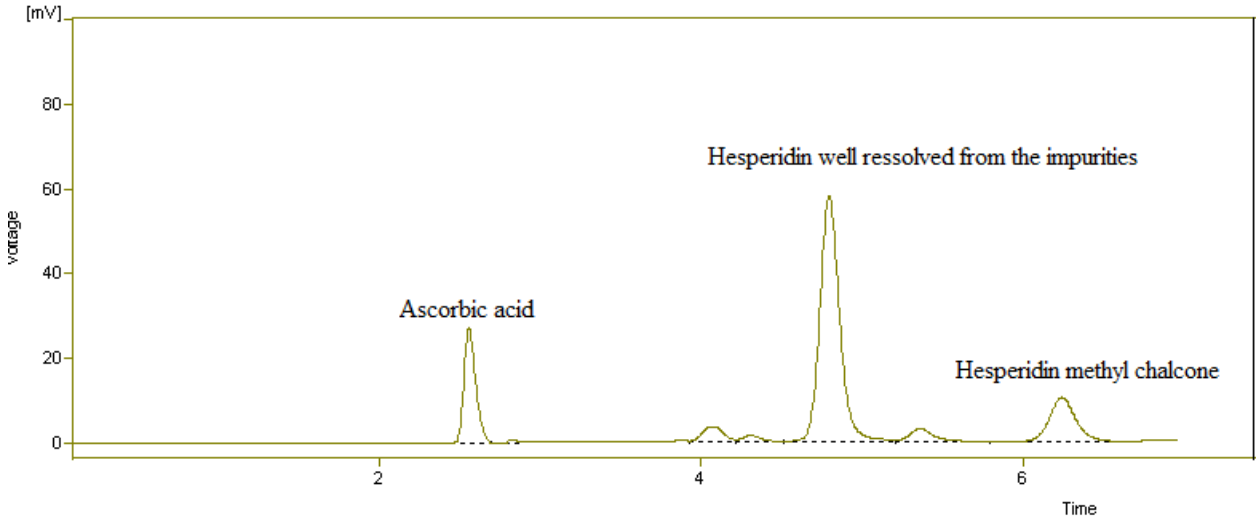

Fig. 12: Chromatographic peak for AA, HP and HMC under optimized condition

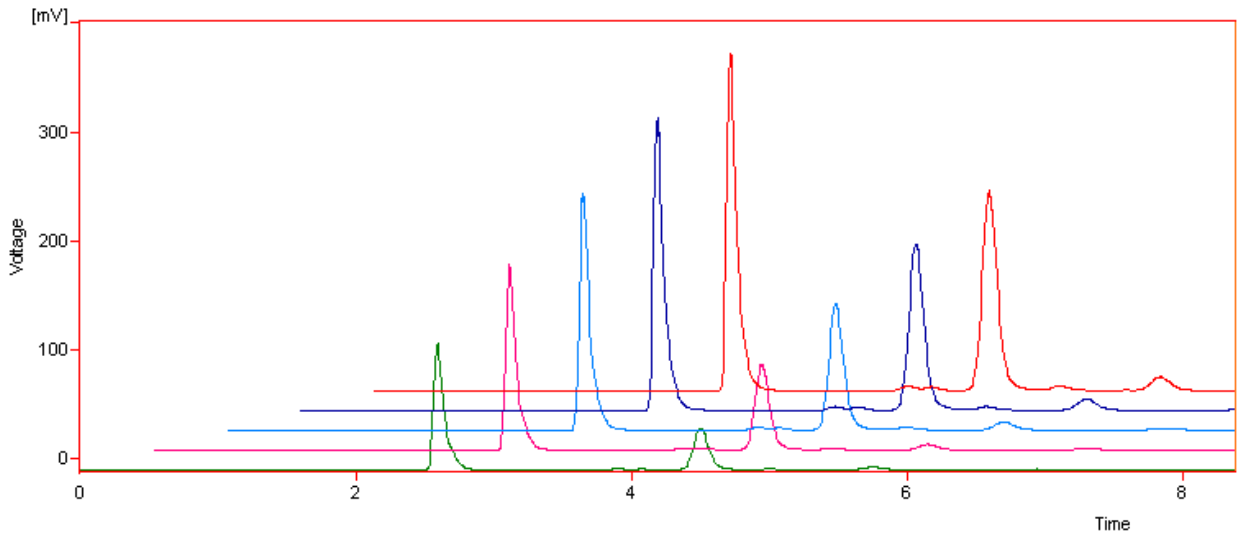

Fig. 13: Overlay chromatogram for AA, HP and HMC

Table 1: Implementation of analytical QbD in pharmaceutical quality system

Sr. no.	Stage wise implementation	Description
1.	Target measurement	Determine what to measure and where/when to measure it. Define Analytical Target Profile (ATP) and develop measurement requirements based on product QTPP (Quality target product profile) and CQA (Critical Quality Attributes).
2.	Select technique	Select appropriate analytical technique for desired measurement defined in ATP. Define method performance criteria.
3.	Risk assessment	Assess risks associated with method input variables, sample variation, and environmental conditions. Risk assessment tools (e.g., FMEA) can be used.
4.	Method development and validation	Examine potential multivariate interactions by DoE and define MODR (Method operable design region) to understand method robustness and ruggedness.
5.	Control strategy	Define control space and system suitability; meet method performance criteria to meet ATP
6.	Continual improvement	Monitor method performance that remains compliant with ATP criteria and thus analysts proactively identify and address the out-of-trend performance of the method. Update with new process and analytical technology.

Table 2: DoE summary: Critical factors and critical responses

CRITICAL FACTORS		
Factor	**Levels**	**Value**
pH	-1	4.0
	0	4.5
	1	5.0
Flow rate (ml/min)	-1	0.8
	0	0.9
	1	1.0
Ratio of ACN (%)	-1	0
	0	5
	1	10
RESPONSES		
1. Asymmetry of Ascorbic acid		
2. Resolution 1 (Resolution between impurity 1 and HP)		
3. Resolution 2 (Resolution between impurity 2 and HP)		
4. Asymmetry of HMC		

Table 3: Criteria selected for optimization

Sr. No.	Response	Selected criteria (Goal)
1.	Asymmetry of ascorbic acid	1.4 to 1.6
2.	Resolution 1	2.0 to 2.7
3.	Resolution 2	2.0 to 2.7
4.	Asymmetry of HMC	0.9 to 1.5

Table 4: Point verification and working point selection

Optimized Solution	Pred. R1	Obs. R1	Pred. R2	Obs. R2	Pred. R3	Obs. R3	Pred. R4	Obs. R4
pH 4.54, F.R. 0.93 & Ratio of ACN 8.88	1.5486	1.690	2.006	2.291	2.0737	1.898	1.0297	1.396
pH 4.37, F.R. 0.83 & Ratio of ACN 9.18	1.589	1.791	2.0665	1.807	2.2134	2.017	1.0097	1.290
pH 4.48, F.R. 0.96 & Ratio of ACN 8.99	1.545	1.601	2.0199	1.910	2.10636	2.275	1.0431	1.463
pH 4.61, F.R. 0.83, Ratio of ACN 9.62	1.5712	1.579	2.0260	1.987	2.29363	2.234	1.00563	1.222
pH 4.7, F.R. 0.87, Ratio of ACN 9.44	1.55761	1.670	2.0147	1.983	2.014	2.297	0.9996	1.193
pH 4.21, F.R. 0.93 & Ratio of ACN 9.98	1.5704	1.474	2.0623	2.253	2.2518	2.446	1.0849	1.188

Table 5: Summary of validation parameters for developed method

Parameter	HMC	HP	AA
Linearity range (µg/ml)	5-25	10-50	25-75
Correlation coefficient	0.9951	0.9991	0.9989
Slope	5.637	29.14	17.42
Intercept	-2.969	15.54	392.2
Intraday precision (%RSD)	1.3739	0.6456	0.7109
Interday precision (%RSD)	1.3366	0.7329	1.3439
LOD (µg/ml)	0.5105	0.3082	1.7946
LOQ (µg/ml)	1.5471	0.9340	5.4384

Table 6: Results of recovery study

% Spiking	C actual (µg/ml)			C added (µg/ml)			C recover* (µg/ml)			% recovery		
	HMC	HP	AA	HMC	HP	AA	HMC	HP	AA	HMC	HP	AA
50	4	12	16	2	6	8	1.94	5.81	8.09	97.00 ±0.75	96.83 ±0.12	101.12 ±0.94
100	4	12	16	4	12	16	3.96	11.5	16.21	99.00 ±1.91	95.83 ±0.91	101.31 ±0.75
150	4	12	16	6	18	24	5.87	17.33	24.61	97.83 ±0.98	96.27 ±1.50	102.54 ±1.01

*Mean value of 3 determinations.

Table 7: Results of robustness study

Factor	Retention time (min)		
	HMC	HP	AA
A. pH of buffer solution			
4.0	5.977	4.617	2.560
4.2	6.243	4.797	2.557
4.4	5.313	4.237	2.553
Mean ± SD	5.844 ± 0.479	4.550 ± 0.286	2.553 ± 0.0035
B. Flow Rate			
0.8	6.853	5.283	2.883
0.9	5.977	4.617	2.56
1.0	5.463	4.207	2.317
Mean ± SD	6.098 ± 0.708	4.702 ± 0.543	2.587 ± 0.284
C. Wavelength			
283	6.022	4.357	2.559
285	6.077	4.683	2.56
287	5.998	4.589	2.458
Mean ± SD	6.032 ± 0.041	4.543 ± 0.168	2.526 ± 0.059

Table 8: System suitability parameters

Parameter	Data obtained*		
	HMC	HP	AA
Retention time (min)	6.0470 ± 0.0413	4.658 ± 0.0435	2.561 ± 0.0049
Theoretical plates per meter	149184± 3736.341	153950.5 ±4496.81	116762.3 ±5009.9
Theoretical plates per column	7459.17 ± 186.412	7617.33 ± 225.004	5838.167 ±250.52
Asymmetry factor	1.263 ± 0.0054	1.268 ± 0.021	1.494 ± 0.037
Capacity Factor	3.837	2.726	1.048
Resolution	3.122 ± 0.173	2.314 ± 0.157	2.969 ± 0.192

*Data obtained is average of 6 determinations ± SD.

Table 9: Applicability of developed method

Formulation : Peridin C tablets			
Labeled claim : HMC:HP:AA (50 mg: 150 mg: 200 mg)			
RP-HPLC method	**HMC**	**HP**	**AA**
	96.2±0.579	98.95±1.129	101.38 ±0.627