Int J Pharm Bio Sci 2015 Oct; 6(4): (P) 227 - 235

Research Article **Analytical chemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

DEVELOPMENT OF A UPLC ASSAY METHOD OF DANAZOL USING QbD APPROACH

MRUNALINI KULKARNI*[1,2] AND VIKAS NAWATHYE[3]

[1] *Maharshi Dayanand College, 25 Dr. S.S.Rao Road,Parel, Mumbai, India*
[2] *JJT University, Jhunjhunu, Rajasthan, India*
[3] *JJT University, Jhunjhunu, Rajasthan, India*

ABSTRACT

A novel stability-indicating reverse phase ultra performance liquid chromatographic method was developed for the estimation of Danazol. To establish specificity and to develop a stability indicating method, Danazol was subjected to the stress conditions like oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Chromatographic separation was achieved on ultra performance liquid chromatography (UPLC) by quality by design (QbD) approach. The eluted compounds were monitored at 270 nm. All the degradation products were well resolved from the main peak, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, precision, linearity, accuracy, robustness and ruggedness.

KEYWORDS: **Danazol, ICH Guidelines, QbD approach, Stability-Indicating method, UPLC**

MRUNALINI KULKARNI
JJT University, Jhunjhunu, Rajasthan, India

INTRODUCTION

Danazol is a derivative of the synthetic steroid Ethisterone[1], a modified testosterone, also known as 17α- ethynyl testosterone[2]. Before becoming available as a generic drug, Danazol was marketed as Danocrine in the United States. It was approved by the U.S. Food and Drug Administration (FDA) as the first drug to specifically treat endometriosis[2]. It is also used in the treatment of fibrocystic breast disease and hereditary angioedema[3]. It acts as antigonadotropin and anterior pituitary suppressant. Danazol has also been used successfully to treat various Diseases associated with autoimmune thrombocytopenia[4.] Danazol is synthetic androgen and is synthesized industrially as an active pharmaceutical ingredient[5]. In general, active pharmaceutical ingredients (APIs) are stored at different conditions depending on their stability. The study of stability of APIs is critical in the drug development process. Many factors can affect the stability of an API, some of them include the manufacturing process, the environmental conditions (such as heat, light and moisture during storage), as well as some chemical reactions such as oxidation, reduction and hydrolysis that might occur[6]. Also trace levels of metallic impurities also can catalyses degradation of APIs during its storage. In the pharmaceutical industry, these degradation impurities formed during stability studies are monitored using different chromatographic techniques like HPLC, UPLC, GC, etc. In the literature, limited LC methods are reported for the estimation of Danazol[7-8]. During stability study, the degradation products may get increased and need to be estimated accurately. Now days QbD approach[9-10] is being used in method development and there are many stability indicating method developments using QbD approach have been published for different products[11-17]. In the present work, forced degradation experiments of the API has been carried out to get the probable degradation impurities and a stability indicating method has been developed on UPLC using QbD approach and the method is validated as per ICH guidelines[18].

MATERIALS AND METHODS

Equipment

The Waters Acquity UPLC system (Waters, Milford, USA) used consists of a pump, auto sampler and a PDA detector. A water bath was used for hydrolysis studies. Photo degradation was carried out in a photo stability chamber. Thermal stability study was performed in a dry air oven.

Chemicals and Reagents

The purity of Danazol used was above 99%, was supplied by Dy Mach Pharma as a gift sample, Acetonitrile and methanol were procured from J.T. Baker limited. High purity water was prepared by using a Millipore Milli Q Plus water purification system (Millipore, Milford, MA, USA).

Experimental

Chromatographic conditions

Tentative HPLC method

Waters Novapak column having dimensions, 150mm x 3.9mm, 4u particle size. Mobile phase used was Water:ACN:MeOH (40:30:30), injection volume 20 µl.

UV detector Wavelength used was 270nm and flow 1ml/min.

Final UPLC method developed using QbD approach

Acquity UPLC, 50mm x 2 mm, 1.7 µm column with mobile phase containing mixture of water, acetonitrile and methanol in the ratio 35:35:30. The UPLC system operated at a flow rate of 0.3 mL/min. The column oven temperature was kept at ambient. The injection volume was 1µl. UV detection was carried out at 270 nm and data acquisition time of 8 minutes. For dilution of sample and standard, mobile phase was used as diluent.

Preparation of stock and sample solutions

A stock solution of Danazol (1 mg/ml) was prepared by dissolving an appropriate amount of the drug in diluent. Working sample solution containing 0.1 mg/ml was prepared from this stock solution for the determination of assay.

Method development by QbD approach

Strategy followed for method development

The development of UPLC method was done with predefined goals, with predefined objectives and by emphasizing on process understanding and control. The ultimate goal of the method development was, to separate degradation products and quantify the main compound. A systematic experimental design was required to understand the in depth knowledge of the method. This was achieved by scouting different components and parameters of the method like column, buffer and mobile phase composition. This generated a database which helped to understand the effect of different variants on the separation of impurities and main compound. More than 45 method conditions were evaluated using the three tiered approach. At the first level, the conditions were evaluated for separation of maximum number of impurities, peaks symmetry, peaks fronting and peaks tailing, etc. This resulted in 20 chromatographic conditions for API. At the second level, these 20 conditions were further evaluated by using more stringent criteria, such as tailing factor should be less than 1.5, peak purity of main compound, etc.The method verification and finalization was done using a risk-based approach as explained in ICH Q8 and Q9 guidelines. This risk assessment was performed to identify the potential risk to the method due to a small change of analytical parameters or under a variety of conditions such as different laboratories, instruments, reagents, days, etc. Based on the results of robustness and ruggedness study an appropriate system suitability criteria was defined to manage risk and ensured the method delivers the desirable method attributes. Based on the results of robustness and ruggedness study the design space was set in which the variation will not affect the quality of the method.

Method Validation experiments

Specificity, precision, linearity, Accuracy, Robustness and Solution stability parameters were verified as part of method validation.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the UPLC method for Danazol was carried out in the presence of its degradation impurities. Stress studies were performed for Danazol to provide an indication of the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted, with a stress condition of Photolytic (1.2 Million Lux hours followed by 200 Watt hours), thermal (105^0C, 3 hours), acid (0.1N HCl, stored at 24hrs), base (0.1N NaOH, stored at 24hrs) and oxidation (3.0% H_2O_2, stored at 24hrs), to evaluate the ability of the proposed method to separate Danazol from its degradation products. Peak purity for the Danazol peak was evaluated by using a PDA detector in all stressed samples.

Precision

The precision of the method was verified by repeatability by injecting six individual preparations of Danazol. %RSD of assay from six preparations was calculated. The same experiment was evaluated by using different instrument, different column and on different day as part of intermediate precision.

Linearity

Linearity test solutions for the method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at 50%, 80%, 100%, 120% and 150% concentration levels & the responses against concentration was checked for all levels.

Accuracy

Accuracy of the method was evaluated using concentration 50%, 100%, and 150% levels of Danazol. Standard addition and recovery experiments were conducted on real sample to determine accuracy. The percentage recoveries for Danazol were calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the cumulative %RSD was checked at different experimental conditions. The flow rate of the mobile phase was 0.3 ml/min, to study the effect of flow rate on the resolution; flow rate was changed by 0.02 units i.e., from 0.28 to 0.32 ml/min. The effect of mobile phase composition was studied by variation in all the three

constituents (Water: ACN:MeOH) of the mobile phase viz. (a) 33:36:31 (b) 35:35:30 and (c) 37:34:29 . Wavelength was varied by ±2 units to check the impact on estimation of Danazol. Further the lot of the column was also changed to check the impact.

Solution stability

Solution stability of Danazol was carried out by leaving sample solutions in tightly capped volumetric flasks for 24hrs at room temperature and RSD of six assay determinations was checked.

RESULTS AND DISCUSSION

Initial method development

The main objective of the chromatographic method was to separate all the degradation impurities from Danazol. An initial degradation study was performed to understand degradation ability of Danazol. After various degradation experiments and analysis on the initial HPLC method it was observed that Danazol mainly degrades in presence of base while under other conditions no significant degradation was observed (Table 1). (Basic and thermal degradation graphs shown in **Figure 1a and 1b).**

Figure 1a&1b

Thermal degradation and NaOH degradation graphs on HPLC

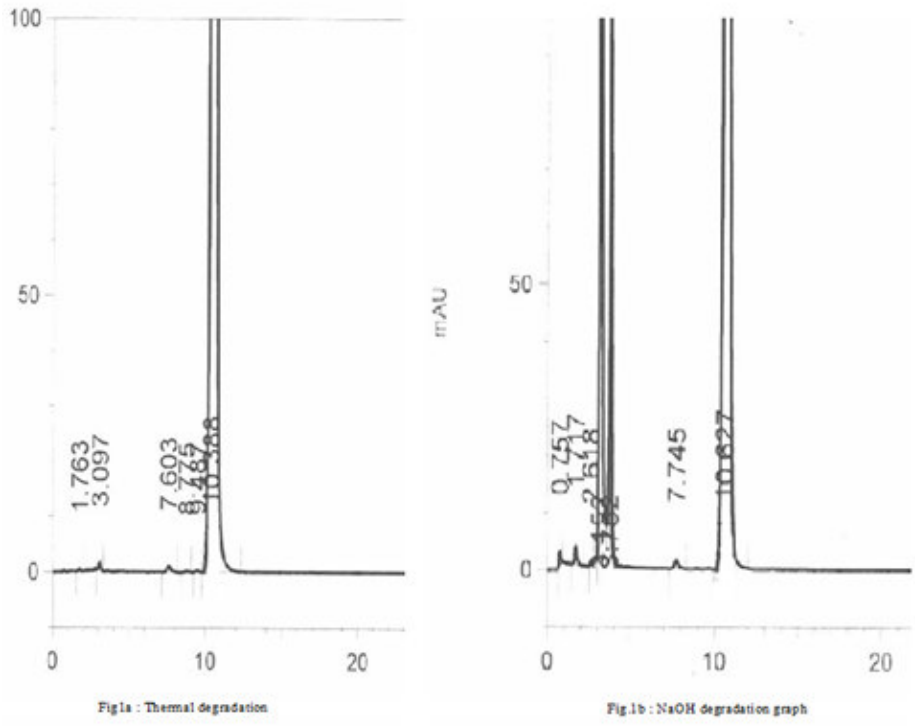


Fig 1a : Thermal degradation

Fig 1b : NaOH degradation graph

Table I

Degradation study observations

Sr. No.	Degradation parameters	Observations
1	Exposed to 0.1N HCl stored at room temperature for 24hrs.	No degradation
2	Exposed to 0.1N NaOH stored at room temperature for 24hrs.	Significant degradation
3	Exposed to 3% H_2O_2 stored at room temperature for 24hrs.	No degradation
4	Heating for 105ºC for 3 hours	Insignificant degradation
5	Exposed to photolytic degradation	Insignificant degradation

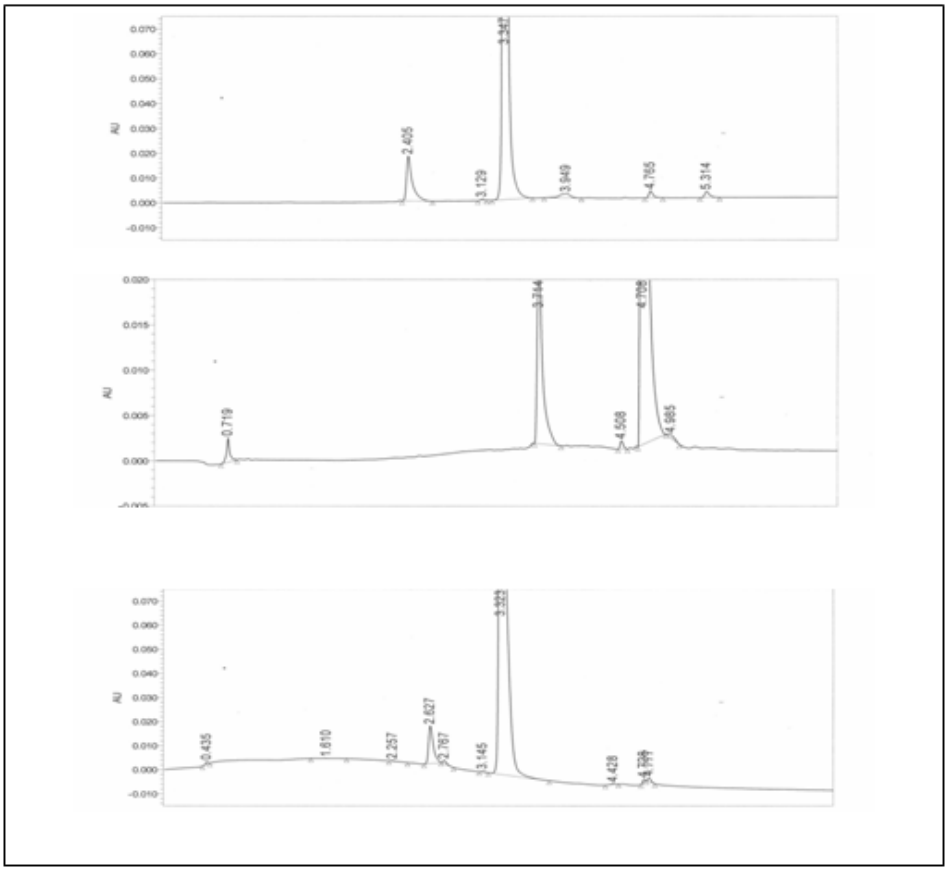
The forcibly degraded sample was then used to develop method on UPLC using QbD approach. Method development on UPLC was initiated to separate degradation impurities from the main compound by changing different analytical components like columns, buffers and mobile phase compositions. Thus more than 45 experiments were carried out. (Table II) (Some of the graphs shown in Figure2).

Table II
Design of experiments

Sr. No.	Analytical components varied	Details
1	Columns	• CSH C18 • BEH C18 • BEH C8 • BEH phenyl
2	Aquaous component of mobile phase	• Water • Ammonium acetate buffer • Ammonium formate buffer
3	Organic component of mobile phase	• Acetonitrile • Methanol
4	Mobile phase combinations	• Water : ACN • Water: Methanol • Water :ACN:Methanol • Buffer:ACN:Methanol
5	Gradient variation of aqueous component	• For water 5% to 70% • For buffer 10% to 60%

After getting a database an appropriate method was selected and validated.

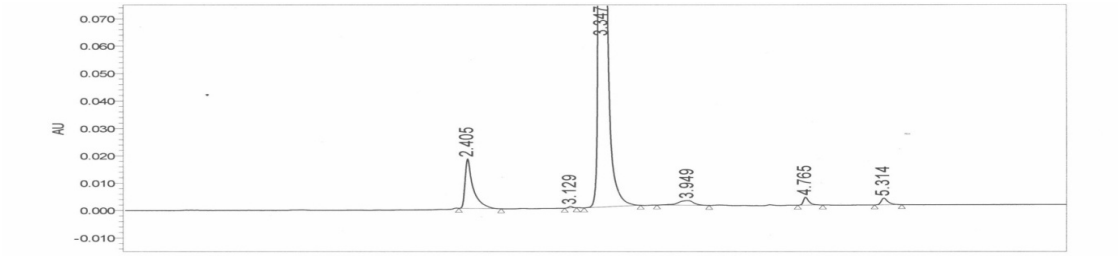
Figure 2
QbD experiments graphs



Method Validation
Specificity

Danazol was found to degrade significantly in basic stress and no degradation or insignificant degradation was observed in photolytic, acidic, thermal and oxidative studies. The typical chromatogram of basic degradation sample is shown in Figure 3. It can be seen that all the peaks due to degradation were well resolved from the peak of Danazol. Photodiode array detector was employed to check and ensure the homogeneity and purity of Danazol peak in the stressed sample solution. The peak purity and separation of possible degradation impurities confirms the stability-indicating power of the method.

Figure 3
NaOH degradation Graph on UPLC method



Precision

The %RSD for the six assay determinations in method precision study was 0.13 %(Table III). This result demonstrated that the method is precise.

Table III
Precision results

Sr.No.	Test name	%Assay
1	Test 1	99.98
2	Test 2	99.81
3	Test 3	99.79
4	Test 4	99.69
5	Test 5	99.97
6	Test 6	99.99
Mean		99.87
Std.Dev.		0.13
%RSD		0.13

Linearity

Linear calibration plot for the assay was obtained over the calibration ranges tested, i.e. 50% to 150% of the specification level. The correlation coefficient obtained was 0.999 for Danazol (Graph presented in Fig. 4), which confirmed good linearity between peak areas and concentration.

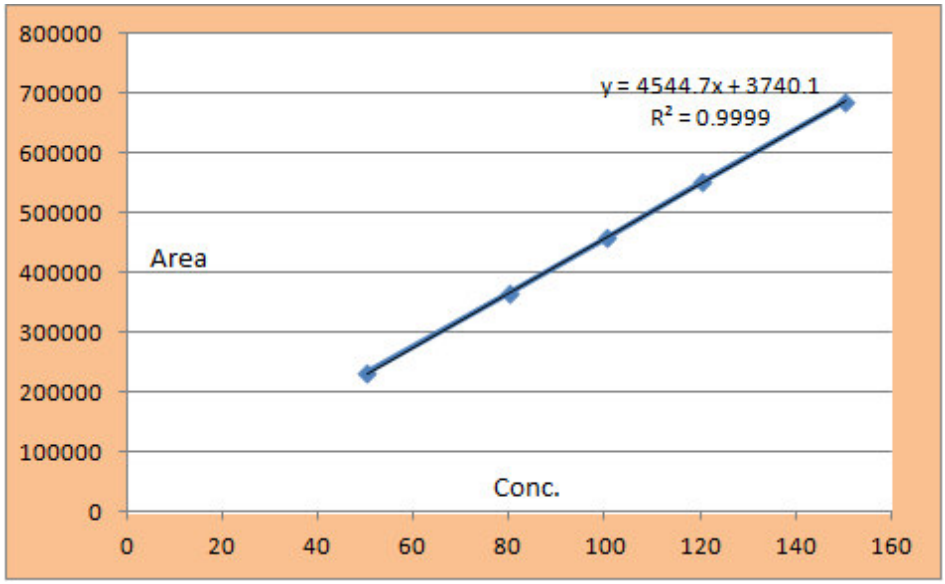


Figure 4
Linearity graph (Concentration Vs Area)

Accuracy

Recovery of Danazol was studied in spiked ranged from 50-150% levels. The % recovery values for Danazol were between 98% to 102%. (Presented in Table IV)

Robustness

In all the deliberate varied chromatographic conditions (flow rate, wavelength and composition of mobile phase), the RSD of assay determinations were observed well within acceptable limit.(Table V)

Solution stability

The cumulative RSD of assay determinations, done with freshly prepared sample set and after storing for 24hrs., observed to be well within the acceptable limit (Table VI). The solution stability experiment data confirms that the solution of Danazol is stable and need not required to be injected freshly prepared.

Table IV
Accuracy results

Concentration Level	Test Name	Quantity spiked (mg)	Quantity recovered (mg)	% Recovery
50%	Test1	50.12	49.70	99.16
	Test2	50.12	49.48	98.72
	Test3	50.12	49.50	98.76
			Mean	98.88
			Stdev	**0.24**
			%RSD	**0.25**
100%	Test1	100.24	99.48	99.25
	Test2	100.24	100.12	99.88
	Test3	100.24	100.00	99.76
			Mean	99.63
			Stdev	**0.336**
			%RSD	**0.34**
150%	Test1	150.36	150.06	99.80
	Test2	150.36	149.63	99.52
	Test3	150.36	149.96	99.74
			Mean	99.68
			Stdev	**0.149**
			%RSD	**0.15**

Table V
Robustness results

Sr. No.	Test name	%Assay Flow 1	%Assay Flow 2	%Assay Wavelength 1	%Assay Wavelength 2	%Assay Composition 1	%Assay Composition 2
1	Test 1	99.96	99.98	99.84	99.98	99.95	99.92
2	Test 2	99.68	99.96	99.68	99.74	99.90	99.46
3	Test 3	99.66	99.73	99.68	99.82	99.81	99.27
4	Test 4	99.69	99.99	99.45	99.63	99.95	99.71
5	Test 5	99.63	99.85	99.89	99.99	99.89	99.55
6	Test 6	99.66	99.83	99.91	99.97	99.69	99.95
Mean		99.71	99.89	99.74	99.86	99.86	99.64
Std.Dev.		0.12	0.10	0.18	0.15	0.10	0.27
%RSD		0.12	0.10	0.18	0.15	0.10	0.27

Table VI
Solution stability results

Sr.No.	Test name	%Assay(Initial)	%Assay after 24hrs
1	Test 1	99.69	99.07
2	Test 2	99.83	99.56
3	Test 3	99.81	99.41
4	Test 4	99.91	99.62
5	Test 5	99.89	99.76
6	Test 6	99.83	99.51
Mean		99.82	99.49
Std.Dev.		0.08	0.24
%RSD		0.08	0.24
Cumulative RSD		0.24	

DISCUSSION

UPLC becomes very prominent in recent years due to its fast approach towards drugmethod development and validation. The smaller particles in column provide not onlyincreased efficiency, but also the ability to work at an increased linear velocity without a loss of efficiency, providing both resolution and speed. This study discusses in detail the QbD approach towards development and validation of Danazol with vital information about its degradation in different stress conditions. The study reveals that the drug is sensitive towards mainly in basic condition. QbD development reveals that the Acquity BEH C18, 100 × 2.1 mm, 1.7 µm column is the appropriate one for this method compared to other columns. According to the van Deemter equation, as the particle size decreases to less than 2.5 µm, the efficiency gains significantly. The small particles in the column play a vital role in gaining the higher efficiency. The robustness and ruggedness study provides sufficient information on the repeatability and reproducibility of the method. Thus in this study a reversed phase UPLC method development approach using QbD principles has been described. First, the method goals were clarified based on the process understanding. The experimental design describes the scouting of the key UPLC method components including column, buffer and mobile phase composition. The interrelationships were studied and the preliminary optimised conditions were obtained for each combination. Here a better understanding of the factors influencing chromatographic separation and greater confidence in the ability of the methods to meet their intended purposes was done. Moreover, this approach provides a thorough knowledge and enables the creation of a chromatographic database that can be utilised to provide alternative method conditions at a future time, whenever the changes to the method are required for the assay of the drug. The new method boosts productivity by providing more information per unit of work as UPLC provides higher resolution, speed, and sensitivity predicted for liquid chromatography. All the validated parameters were found within acceptance criteria.

CONCLUSION

The validated method is specific, linear, precise, accurate, robust, rugged and stable for 24hrs and can be applied for the assay determination of Danazol. The drug is stable in acidic, oxidative, thermal, photolytic and hydrolytic conditions but unstable in basic condition. The potential of QbD approach gives the base for simultaneous development of multiple methods including impurity methods, assay method, dissolution method, and cleaning validation method, and thus it should be implemented.

ACKNOWLEDGEMENT

We thank Dy Mach Pharma for the gift sample of Danazol and also UGC for the financial aid given for this research work.

REFERENCES

1. Mousavi S R, Mousavi S M, Samsami M, Mahdikhah Z, Comparison of Tamoxifen with Danazol in the management of fibrocystic disease, International Journal of Medicine and Medical Sciences, 2(10), 329-331, (2010).
2. Cottreau C M, Ness R B, Modugno F, Allen G O, Goodman M T, Endometriosis and its treatment with Danazol or Lupron in relation to ovarian cancer , Clinical Cancer Research, 9, 5142-5144, (2003).
3. Farkas H, Czaller I, Csuka D, Vas A, Valentin S, Varga L, Szeplaki G, Jakab L, Fust G, Prohaszka Z, Harmat G, Visy B, Karadi I, The effect of long-term Danazol prophylaxis on liver function in hereditary angioedema –a longitudinal study, Eur J Clin Pharmacol, 66, 419–426, (2010).
4. Alvarez G C, Marina V M, Mendez R J, Buitimea A L, Roman M B, Gomez R C, Esparza J R, Fragoso L R, Combined therapy with Danazol, pegilated interferon, and ribavirin improves

thrombocytopenia and liver injury in rats with fibrosis, Annals of Hepatology, 6(4), 233-241, (2007).
5. The Merck Index, Published by Merck Research Laboratories, NJ USA, Fourteenth edition, 474, (2006).
6. ICH guidelines Q1
7. The United States Pharmacopeia, USP-36, 2, 3140, (2013).
8. Prajapatia H N, Patela D P, Patela N G, Dalrympleb D M, Serajuddina A T M, Effect of difference in fatty acid chain lengths of medium-chain lipids on Lipid/Surfactant/Water phase diagrams and drug solubility, J. Excipients and Food Chem., 2(3), 73, (2011).
9. International Conference on Harmonisation (ICH) Tripartite Guideline, ICH Q8(R2), (2009).
10. Riddhi Patel, Om Bagade, Bindiya Patel, Kuldeep Ramteke, Downright and apparent appraisal on Quality By Design: A goal-oriented approach, Int J Pharm Bio Sci., 5(4), 607-626, (2014).
11. Raghavaraju T V, Rajakumar S, Rao I M, Rao N S, Development and validation of a stability indicating hplc method for the estimation of Rabeprazole impurities in pharmaceutical dosage forms by design of experiments, Asian J Pharm Clin Res, 6(4), 43-51, (2013).
12. Xavier C M, Pavagada J R, Kanakapura B V, Hosakere D R, RP-UPLC method development and validation for the determination of Nateglinide in bulk drug and pharmaceutical formulations: a quality by design approach, Malay J Pharm Sci, 10(1), 23–44, (2012).
13. Bhatt D A, Rane S I, QbD approach to analytical RP-HPLC method development and its validation, Int J Pharm Pharm Sci., 3(1), 179-187, (2011).
14. Karmarkar S, Garber R, Genchanok Y, George S, Yang X, Hammond R, Quality by Design (QbD) Based Development of a Stability Indicating HPLC Method for Drug and Impurities, Journal of Chromatographic Science, 49, 439-446, (2011).
15. Thummala V R, Seshadri R K, Tharlapu S J, Ivaturi M R, Nittala s R, Development and validation of a UPLC method by the QbD-approach for the estimation of Rabeprazole and Levosulpiride from capsules, Sci Pharm., 82, 307–326, (2014).
16. Shah A d, Patel C N, Implementation of QbD approach to the RP-HPLC Method development and validation of Roflumilast in bulk and tablet dosage form: an application in degradation study, World Journal of Pharmacy and Pharmaceutical Sciences, 3(6), 2281-2307. (2014).
17. P. Srinivas, T Siva Rao, Elaiyalvar Ramakrishnan, D V Gowda, Development and validation of analytical method for the estimation of Omeprazole, Domperidone and their related substances, Int J Pharm Bio Sci., 4(2), 906-911, (2013).
18. International Conference on Harmonisation (ICH) Tripartite Guideline, ICH Q2(R1), (1994).