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Quality by Design (QbD) approach towards the development and validation of HPLC method for Gentamicin content in biodegradable implants

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ABSTRACT

A High performance liquid chromatography (HPLC) method for quantitative analysis of Gentamicin is developed using a Quality by Design (QbD) a statistical approach. Gentamicin Sulphate is broad spectrum antibiotic aminoglycoside drug. It is used to treat infections and inflammatory diseases caused by susceptible organisms. Due to regulatory needs QbD has gain magnitude. Foremost target profile is determined and then qualification of instrument is done prior to initiation of actual study. Chromatographic separation is achieved on a C-18 column (250 × 4.6mm, 5micron). The mobile phase used is isocratic elution system consisting of methanol and 15mM di ammonium hydrogen phosphate buffer (pH 10.00) in the ratio 70:30 v/v. In development of HPLC method factors like flow rate, mobile phase composition, column temperature and wavelengths are critical to maintain. Hence Plackett Burman design was used as screening model. Further Box Behnken model was applied as optimization model for the interaction and quadratic effects of three factors namely temperature of the column, flow rate, and wavelength on the selected responses. Effect of these parameters is studied on USP tailing (response). Tailing less than 1.2 was considered as desirable. Results are analyzed using surface diagrams. Verification of the software generated result is done by taking six replicates of the run. Finally the method was validated as per ICH Q2 guidelines. QbD approach is successfully applied for HPLC assay method development of the Gentamicin sulphate.

Keywords: QbD, HPLC, Gentamicin, Box Behnken design, Plackett Burman design.

INTRODUCTION

The aminoglycoside antibiotic Gentamicin has a broad spectrum activity against both the gram positive and gram negative bacterial infections [1]. It is originally obtained from the micro-organism Micromonospora purpurea by fermentation process and is commercially available in different forms of medicine to combat different bacterial infections in humans and cattle [2-5].Gentamicin is highly water soluble, highly polar, and non-volatile and lacks a UV chromatophore. These physical and chemical properties of Gentamicin are a major challenge to retain and separate them through RP-HPLC method. There are different direct and indirect HPLC methods reported in the literature so far for the detection of Gentamicin [5-27].The direct methods comprise refractive index (RI) detection [6], Evaporative Light Scattering Detection (ELSD) [7, 8], Electrochemical Detection (ECD) [9-11], charged aerosol detection (CAD) [12] and mass spectrometry [13-14] and the indirect methods comprise either pre- or post-column derivatization [15-20]. All the above methods have their own advantages as well as limitations such as incompatibility of RI detection with gradient methods, cumbersomeness of pre-column derivatization method and chance of electrode poisoning and very high sensitivity in ECD method. Though there are various HPLC methods available in the literature, they are time consuming and costly for routine analysis of sample. As per ICH Q8 (International Conference on Harmonisation of Quality 8), Quality by design (QbD) is a systematic approach to pharmaceutical method development that begins with predefined objectives and emphasizes product, process understanding and process control based on sound science and quality risk management [27]. Development of various HPLC methods for the analysis of drug substances and drug products using QbD approach are widely

reported in the literature [28-42]. In this paper, we discuss the detection and separation of Gentamicin (sulphate) for the first time in biodegradable implants by RP-HPLC method through QbD approach. This method is simple, reliable, cost-effective, selective, sensitive and robust. Plackett Burman design was used for screening of factors which affect response and the factors that have significant effect on response were selected for optimization by Box Behnken experimental design.

MATERIALS AND METHODS

All the organic solvents used for experimentation were of HPLC grade. Chromatography grade methanol (%) and diammonium phosphate (%) were purchased from Merck Millipore. All the aqueous solutions were prepared using Milli-Q water. Reference standard of Gentamicin was obtained from Food, Drugs and Chemicals (FDC) Limited, Aurangabad, Maharashtra, India.

Instrumentation and the chromatographic conditions

RP-HPLC of Gentamicin was analyzed using Agilent HPLC system equipped with PU 2089 quaternary gradient pump, UV-2075 plus detector, LC-Net II/ADC communication module and chromatographic separation was achieved on X-Terra RP C-18 column (150 x 4.6mm, 5 µm packing). Data analysis was carried out using Empower 2 software build 2154 SPs version 1.8.6.1. The gradient elution system consisting of methanol and 0.01m diammonium hydrogen phosphate buffer was used as mobile phase. Here the proportion of mobile phase was changed based on the experimental design.

Extraction of Gentamicin from Implants

Gentamicin from polymer based implants was extracted via liquid-liquid extraction technique using water-DCM (Dichloromethane) as a solvent mixture. Gentamicin is freely soluble in water and insoluble in DCM. But remaining polymer blend was miscible in DCM. Triplicate washing was given to the DCM extract. Sample concentration was made as accordingly.

Gentamicin sample preparation

A stock solution of 10mg/mL Gentamicin was prepared in water and then, different concentrations of 250-10,000 ppm were prepared by serial dilution method.

Analytical target profile

The target profile is a prospective summary of the quality characteristics of a drug product that will be ideally achieved to ensure the desired quality and standard [27]. Here, the main aim of the RP-HPLC method of Gentamicin is to be robust, sensitive, accurate and precise with USP tailing less than 1.3, analysis time less than 10 min. A robust method should be developed with the help of visualizing a design space as per the QbD norms.

Risk assessment

Here, Pareto analysis is studied for said method by software generated results (Figure 1). In Pareto chart, 6 dummy factors can be seen which are not real factors and any changes in those factors do not affect the system and response.

Method design

Screening designs are used to sort out the most significant factors from the potentially influencing factors which greatly affect responses. They are applied in the circumstance of optimizing separation techniques during screening, testing of robustness and in the context of optimizing formulations, products or method. Here, Plackett Burman design was used to find significant factors affecting the response [43-45]. For that three level designs was applied for five factors that are flow rate, injection volume, column oven temperature, detection wavelength, and methanol concentration in mobile phase. Experimental conditions are given in Table 1.

Table 1: The response variables for Chromatographic factors in Plackett–Burman experimental design of Gentamicin

Sr.No.	Chromatographic factors	Level used	
		Low	High
A	Flow rate (mL/min)	0.4	0.8
B	Detection wavelength (nm)	255	259
C	Column temperature (°C)	38	42
D	Injection volume (µL)	38	42
E	Methanol concentration (%)	25	35

For optimization response surface methodology was used with five runs of centre point (Table 2). Factors selected were injection volume, column temperature and detection wavelength and the results of Plackett Burman design

helped to sort these factors. Evaluations of main factors and their two factor interaction on peak USP tailing factor were done. Injection volume and methanol concentration were kept constant at 40°C and 30 % respectively.

Table 2: Three levels for Box Behnken of three factors

Chromatographic conditions	Level used		
	Low	Centre	High
Flow rate (mL/min)	0.3	0.6	0.9
Wavelength (X3) (nm)	254	257	260
Column temperature (X2)(°C)	37	40	43

Experiments were conducted by making runs of the standard Gentamicin solution in HPLC and the average of USP tailing (Fig 3). It was analysed using Design Expert 8 software and the application of two factor interaction analysis fitted well for the model. $Y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta12X1X2 + \beta13X1X3 + \beta23X2X3$, where Y is the response, β0 is the arithmetic mean response, β1, β2 and β3 are regression coefficients of the factors X1, X2 and X3, respectively. β12, β13, β23 are interaction coefficients [43-45].

Table 3: Box Behnken design used for study

Run	Coded (X1, X2, X3)	Injection volume(µL)	Column temperature (°C)	Wavelength (nm)
1	+0+	0.9	257	43
2	0-+	0.6	254	43
3	000	0.6	257	40
4	0+-	0.6	260	37
5	++0	0.9	260	40
6	000	0.6	257	40
7	-0+	0.3	257	43
8	--0	0.3	254	40
9	00-	0.9	257	37
10	+-0	0.9	254	40
11	-0+	0.3	257	37
12	0++	0.6	260	43
13	000	0.6	257	40
14	000	0.6	257	40
15	0--	0.6	254	37
16	000	0.6	257	40
17	-+0	0.3	260	40

(Where '+' indicates the high value, '-'indicates lower value and '0' is the centre)

Critical Quality Attributes (CQA)

CQA can be set from risk assessment by Pareto analysis as given in ICH Q9 guideline [30]. A critical factor which affects the tailing was determined. Factors such as flow rate, column temperature, and detection wavelength were found to be critical.

Validation

As per International Conference on Harmonization of analytical validation (ICH Q2) guidelines, linearity, range, accuracy, precision, and robustness of the optimized chromatographic method was validated [44]. For system suitability, standard solution of 500 µg/mL of Gentamicin in water was prepared. Before sample analysis six replicate standard solutions were analyzed. The acceptance criteria for Gentamicin was than 2% relative standard deviation (RSD) for peak area, retention time, symmetry and USP tailing factor less than 2.

Linearity

Standard calibration curves were prepared with six different concentrations in the range of 250-1000 µg/mL. By injecting each concentration in triplicate, linearity of Gentamicin over the concentration range was determined. Linear calibration curves of drug concentration versus peak area were plotted using linear least squares regression and evaluated for linearity.

Accuracy and precision

Accuracy and precision of the method were evaluated for Gentamicin by analyzing standard samples prepared from stock solution. As a part of validation and quality control, three replicates each of high (5000µg/mL), intermediate (1000µg/mL) and low (250µg/mL) concentrated standards were analyzed for three consecutive days. Accuracy and precision were determined by analyzing the average, RSD of the peak areas and their resultant concentrations. An acceptance criterion for the precision is that the relative standard deviation of the standards should not be more than 2%.

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. An analysis should be reliable with respect to deliberate variations in method parameters such as wavelength (+ 2 nm), column temperature (+ 2 °C) and flow rate (+ 0.2 mL/min).

RESULTS

Mobile phase combination also has to be considered while the optimization of retention time and then separation was carried on: X-terra RP C18 column (250 x 4.6mm, 5 µmpacking) with mobile phase of 15mM diammonium phosphate buffer: MeOH at 70:30 v/v ratio and pH value of 10.00. Peak was obtained at retention time of 5.4 min, at flow rate of 0.6 mL/min and column oven temperature of 40 °C. Further optimization was done by carrying runs as by Box-Behnken model. Five factors were analyzed by Plackett Burman design and the significant factors were pointed out for the optimization of method factors such as wavelength, column temperature and flow rate. Multivariate regression analysis was applied and fitted two factor interaction model was obtained for the USP tailing factor of peak. Regression analysis and p-values were obtained from the software as shown in Table 4.The analysis of variance (ANOVA) and the effect of interaction terms on the USP tailing of the peak were studied to note the significance of the factors. The p-values supported to assert that the results as 'statistically significant' by convention and $p<0.05$.

DISCUSSION

A value of prob>F of the model and was less than 0.05, hence model was found to be significant, (prob>F = 0.0056). Model used was accurate with R2 of 0.892 and a lack of fit was not found to be statistically significant. Significant factors found were wavelength (p-value 0.0035), column temperature (p-value 0.0079) and interaction of flow rate vs. wavelength (p-value 0.0197).

An inverse relationship has been noticed for the plot of flow rate and wavelength vs. tailing. The response surface and counter plot were studied and plotted in 3-D graph format as given in Figure2. The effect of wavelength and flow rate on tailing was clearly noticed from the graph as shown in figure 2a where tailing was found to increase at higher wavelength (257 nm) and decrease at lower wavelength and was optimum at flow rate of 0.8-0.9. Also, the effect of column oven temperature and flow rate on tailing was studied from the plot figure 2b where tailing was less at column temperature 38-39°C, within the limit at 37-40 °C and out of specified limit above wavelength 257 nm as shown in figure 2c. The critical response was set to minimize tailing below target value of 1.3.The optimum condition chosen from the obtained runs were 257nm wavelength, 0.9 mL/min flow rate, 37 °C column temperature and 1.17 tailing (figure 3).

Table 4: Regression coefficients and associated probability values for the USP tailing of Gentamicin

Term	Coefficient	p-value
Box Behken Model	1.314706	0.0056
Flow rate (mL/min)	-0.0125	0.2485
Wavelength (nm)	-0.03875	0.0035
Column temp (°C)	0.03375	0.0079
Flow rate x wavelength	-0.04	0.0197
Flow rate x column temp	0.015	0.3229
Wavelength x column temp	0.0225	0.1499

Pareto chart

Significant factors in Pareto chart are A: Flow rate B: wavelength and C: column temperature. Other seven factors are dummy factors which do not have any physical effect on response and merely added to complete the database of software.

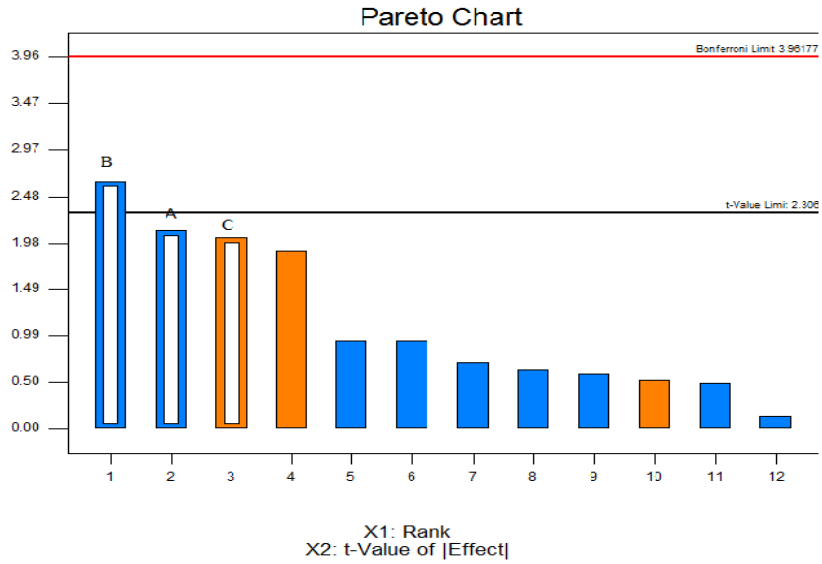
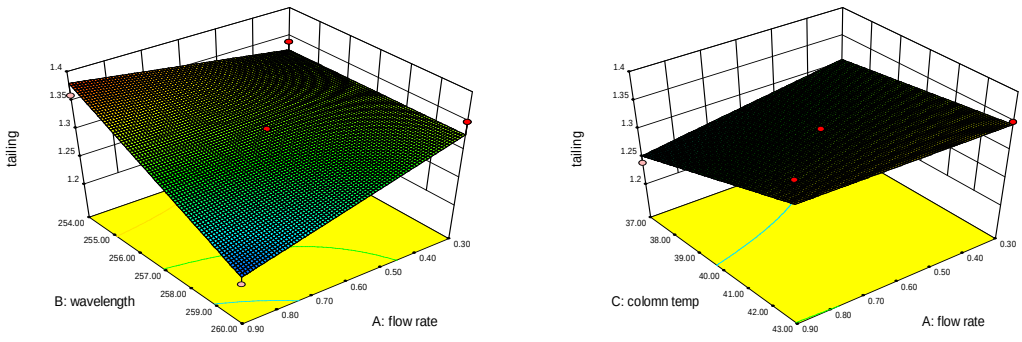
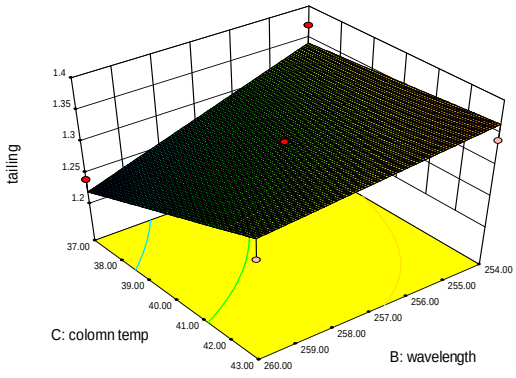


Figure 1: Pareto chart for Critical Quality Attribute (CQA), USP tailing factor of Gentamicin



(a) Effect of wavelength & flow rate **(b) Effect of column temperature & flow rate**



(c) Effect of column temperature and wavelength

Figure 2: Response surfaces (3D) and contour plots showing the effects of wavelength, flow rate and column temperature on USP tailing factor of Gentamicin

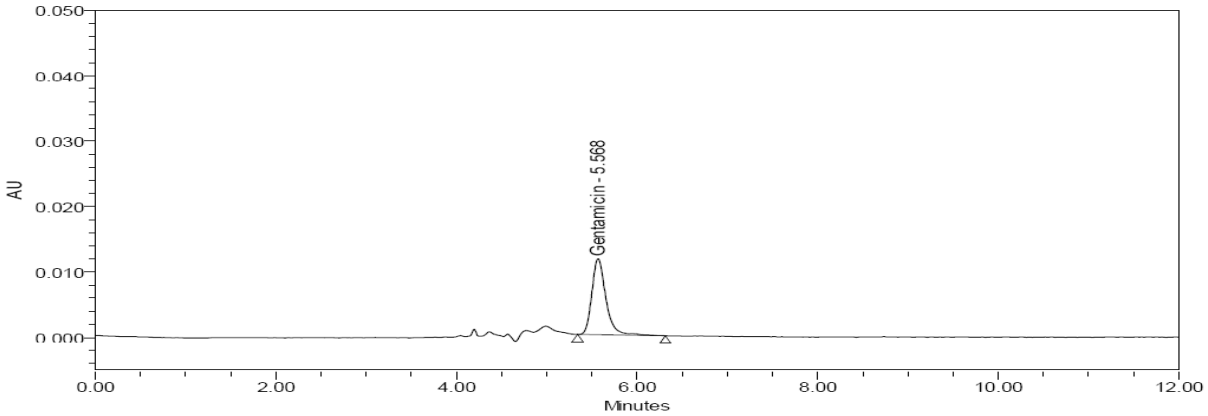


Figure 3: Representative chromatogram of Gentamicin with optimized conditions Method validation

Method validation was done according to ICH guidelines Q2 [46] and the results were within the specified limit. Thus the developed method is found to be accurate, sensitive, and robust. Validation results are given below in (Table 5, 6, 7) and (Figure 3).

Table 5: Validation of method in terms of Accuracy and Precision

Level	Average area	%RSD
Intra-day	123462	1.2
Inter-day	123455	1.1

Table 6: Linearity of Gentamicin

Standard Concentration (µg/mL)	Peak area of Gentamicin
250	14563
500	31529
1000	62315
2000	121196
5000	324015
Regression equation	Y = Mx + C
Regression coefficient	R2 =0.999

Table 7: Validation in terms robustness for Gentamicin

Sr. No.	Variables		Retention time	Area of peak
1	**Wavelength (nm)**	+2	5.43	124186sss
		0	5.3	125491
		-2	5.39	125375
		average	5.33	125452.3
		% RSD	0.974	0.053
2	**Flow rate (mL/min)**	+0.2	6.534	108838
		0	4.699	109288
		-0.2	6.534	109211
		average	4.66	109262.3
		% RSD	1.44	0.0406
3	**Column temperature (°C)**	+5	5.356	126404
		0	5.511	126718
		-5	5.468	126833
		average	5.530	126794.7
		% RSD	1.19	0.052

CONCLUSION

In this paper a quality by design (QbD) approach was applied to the fast, robust and reliable HPLC method with the assistance of the latest statistical methods. The method development consists of complete understanding of intended purpose. Method was successfully passed through validation and has been used regularly and trouble free. Elements of Quality by Design (QbD) like analytical target profile, instrument qualification, risk assessment, experimental design was studied. Final method conditions are set at injection volume of 20 µL, column temperature at 40°C and wavelength of 257nm and flow rate of 0.6mL/min. Mobile phase was set as 0.01 m diammonium hydrogen phosphate and methanol at 70:30 v/v ratios. Quality by Design (QbD) approach is successfully applied to HPLC method development of Gentamicin content in implants.

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REFERENCES

[1] R.S. Edson, C.L.Terrell, The aminoglycosides: streptomycin etc. Mayo Clin. Proc.74, **1987**; 62 (10) , 916-920.
[2] M.J.Weinstein ,G.M. Luedemann , E.M. Oden ,G.H. Wagman, Antimicrobial Agents and Chemotherapy, In Proceedings of the Third Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington DC. **1963**, 1–13.
[3] H.W. Gearald, M.O.Edwin ,J.W. Marvin, *App. Microbiology*,**1968**; 16(4J) , 624-627.
[4] The United States Pharmacopoeia and the National Formulary, USP23, NF18,**1995**, 702.
[5] Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th ed, **2002**, 1114.
[6] D.Samain , P. Dupin, P.Delrieu, G.Inchaups, *J Chromatographia*, 24, **1987**, 748–752.
[7] S. Karolina ,B. Agata , F. Zbigniew ,S. Katarzyna, *J Chromatographia*, **2010** , 1225–1229.
[8] N.C. Megoulas, M.A.Koupparis, *J Pharm. Biomed. Anal*. **2004** , 36 , 73–79.
[9] C.Shruti, M.Vicky , H. Jos , A. Erwin, *Analytical Methods*., **2013**,5, 2491-2496.
[10] E. Adams ,W.Roelants, R.De Paepe , E.Roets, J. Hoogmartens, *J Pharm. Biomed. Anal.*, **1998**,18, 689–698.
[11] R.T. Parfitt, D.E.Games, M.Rossiter, M.S.Rogers, A.Weston, *Biomed. Mass Spectrom.*, **1976** , 3, 232–234.
[12] T.A.Getek, M.L.Vestal, T.G.Alexander, *J. Chromatographia*.,**1991**,554, 191–203.
[13] M.Cherlet, S.De Baere, P.De Backer, *J. Mass Spectrom.*,**2000**,35,1342–1350.
[14] D.Loffler,T.A. Ternes, *J. Chromatographia A*., **2003**,1000 ,583–588.
[15] M.C.Lecaroz, m.A.Campanero,C.Gamazo, M.Blanco-Prieto, *J. Antimicrob. Chemother.*, **2006**, 58 , 557–563.
[16] S.K.Maitra ,T.Y.Yoshikawa, J.L.Hansen,I.Nilsson-Ehle, W.J.Palin,M.C.Schotz, L.B.Guze, *J. Clinical Chemistry*., **1977**, 23 , 2275–2278.
[17] G.W.Peng, M. A.Gadalla, A.Peng, V.Smith, W.L.Chiou, *Clinical Chemistry*,**1977**, 23,1838–1844.
[18] W.Lesniak ,J.Mc Laren, W.R.Harris ,V.L.Pecoraro,J. Schacht,*Carbohydrate research.,* **2003**,2853–2862.
[19] A.Olajire, Adegoke, *African J of Pure and Appl Chem.*, **2012** ,129-140.
[20] L.O.White , A.Lovering , D.S. Reeves, *Ther.Drug Monit.*, **1983**,5,123–126.
[21] I.Nina ,L. Eran , S.Stefan, *Antimicrobial Agents Chemotherapy*.,**2000**,44,1443–1447.
[22] B. A. Elisabete de Almeida, R.L.Felipe,A.P.Terezinha de Jesus, *Brazilian J of Phar*.,**2011** , 262-268.
[23] S.Karolina ,B.Agata, F.Zbigniew, S.Katarzyna, *Chrom1qa*., **2010**,1225–1229.
[24] A.O.Mahmoud ,M.N.Dalia, A.H.Mohamed, A.A. Alshymaa, *J of App Pharm.*, **2013**, 151-161.
[25] M.L.Marie-Paule, G.Youri,M.T.Paul,*Antimicrobial Agents Chemother.*, **1999**,727–737.
[26] E.Kaale , S. Leonard, S. Van, E.Roets , J.Hoogmartens, *J Chrom A.*, **2000**, 895, 67–79.
[27] ICH Q8 Pharmaceutical Development, ICH Q9 Quality Risk Management, ICH Q10 Pharmaceutical Quality System.
[28] P.F.Gavin, B.A.Olsen , *J. Pharm Biomed Anal.*, **2008**,46 ,431– 441.
[29] W. Li , H.T. Rasmussen, *J. Chromatographis A.*, **2003**,1016 ,165–180.
[30] Y.H.Lin, Y.H.Yang,S.M. Wu, *J. Pharm Biomed Anal.*, **2007**,44,279–282.
[31] S. Karmarkar,R.Garber,Y.Genchanok, S.George, X.Yang, R.Hammond, *J. Chro Sci*., **2011**, 49, 439-446.
[32] S. Fekete ,J. Fekete,I. Molnar, K.Ganzler, *J. Chromatographia A.*, **2009**, 1216 ,7816–7823.
[33] B.J. Stojanovicn, T. Rakic,B. Slavkovic, N.Kostic, A. Vemic, A. Malenovic, *J. of Pharm Analysis.*, **2013**, 3 , 45-52.
[34] A.H. Schmidt, I. Molnar, *J. Pharm Biomed Anal.*, **2013** , 5 , 78-79.
[35] K. Garg ,V. Reddy, S. Sait , T. Krishnamurthy, J. Vali, M. Reddy, *J. Chromatographia*, **2013** , 76 ,1697-1706.
[36] E. Rozet, P. Lebrun, B. Debrus, B. Boulanger,P. Hubert, *Trends in Anal Chem.*, **2013**, 42 ,157-167.
[37] P. Ramalingam, B.Kalva, R.Yiragam, R. Padmanabha, *Int J of Anal Chem.*, **2015** , Article ID 868727.
[38] S. Karmarkar,R. Garber,Y. Genchanok, S. George, X. Yang, R. Hammond, *J. Chrom Sci.*, **2011**, 49 , 439.
[39] F. Vogt, A.Kord, *J. Pharm Sci.*, **2011**, 100, 797-812.
[40] B. Debrus, D. Guillarme, S. Rudaz, *J. Pharm Biomed Anal.* **2013**, 84, 215-223.
[41] B. Dejaegher, Y. Vander Heyden, *J. of Pharm Biomed Analysis.*, **2011**, 56,141–158.
[42] D. Awotwe-Otoo,C. Agarabi, P.J.Faustino,M.J. Habib,S.Leec,M.A. Khana,R.B. Shaha, *J. Pharm Biomed Anal.*, **2012** , 62 , 61-67.
[43] L. George, HPLC Methods for recently approved pharmaceuticals, Wiley & Sons : New York, **2005**.
[44] A. Pedro, *J. of Chrommatographia B.* **2009** , 877, 2224–2234
[45] G. Mark, *Anal. Chemi*stry,**1996**, 68 (9), 305–309.
[46] ICH Q2 : Vaidation of Analytical Procedures, Pharmaceutical Quality System. GMP Publ. **2005**.