International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Using RP-HPLC Method for Simultaneous Determination of Fructose and Mannitol in Anidulafungin for Injection

Bommana Sahithi[1], Dr. M. Sunitha Reddy[2]

[1]Student, Department of Pharmaceutical Analysis, Jawaharlal Nehru Technological University Hyderabad, JNTUH

[2]Professor, Department of Pharmaceutical Analysis, Jawaharlal Nehru Technological University Hyderabad, JNTUH

Abstract: ***A simple, selective, precise and stability-indicating high-performance liquid chromatographic method for the development and validation of an analytical method for the determination of fructose and mannitol in anidulafungin injection using RP-HPLC, a simple, specific, sensitive, accurate, precise and reliable method was developed and validated. The method was created by utilizing acetonitrile and water as the mobile phase in isocratic pump mode. We use a Shodex ashipack amino column (250 × 4.6 mm,5µm) for separation, which effectively separates fructose and mannitol in the drug product. The flow rate is 1mL/min, and we use RI detection with a detector sensitivity of 512.The retention time of fructose and mannitol were found to be 10.27, 12.36 min respectively in Anidulafungin for injection. After validation, the optimized method met acceptance criteria for specificity, linearity, LOD and LOQ prediction, accuracy, precision, reliability, and stability when tested against the solution. Investigations into the stability of samples. The method outlined above is suitable for conducting daily routine analysis.***

Keywords: RP-HPLC, Anidulafungin, Fructose, Mannitol, RI detector, pharmaceutical analysis

1. Introduction

Sugars have many roles, in all aspects of plant life, as structural materials, respiratory substrates for the generation of energy and metabolic intermediates, and in the synthesis of macromolecules and other cell constituents. Sugars promote the expression of enzymes in connection with the biosynthesis, utilization, and storage of reserves (including starch, lipids, and proteins) and regulation the expression of various genes. Glucose and other carbohydrates have been reported to increase production of secondary metabolites and proteins in fungi, which could be attributed to the rapid utilization of the preferred carbon sources.

Anidulafungin is a semisynthetic echinocandin antifungal lipopeptide (cyclo hexapeptide with lipophilic acyl side chain) synthesized from a fermentation product of Aspergillus nidulans. It has activity against pathogenic fungi including Candida spp. AnidulafunginIt is approved by FDA in the year 2006 for the treatment of Esophageal Candidiasis.The echinocandins, and structurally related pneumocandins, inhibit the enzyme β-1,3-D-glucan synthetase. β-(1,3)D-Glucan,a polymer of glucose, is an integral component of the fungal cell wall. Chemically it is N-[(3S,6S,9S,11R,15S,18S,20R,21R,24S,25S,26S)-6-[(1S,2S)-1,2-dihydroxy-2-(4-hydroxyphenyl)ethyl]-11,20,21,25-tetrahydroxy-3,15-bis[(1R)hydroxyethyl]-26-methyl-2,5,8, 14,17,23-hexaoxo-1,4,7,13,16,22-hexazatricyclo[22.3.0.0^{9,13}]heptacosan-18-yl]-4-[4-(4-pentoxyphenyl)phenyl]benzamide.

The molecular formula of Anidulafungin is C58H73N7O17 and molecular weight is 1140.2369. It is white to off-white powder which is available in lyophilized form which has to be reconstituted with 30 mL of water during its use. It is freely soluble in methanol and its solubility is about 0.0564 mg/ml. The plasma half-life of the drug is 40-50 h. The pKa value for Anidulafungin is 9.46 for strongest acid and -3.5 for strongest basic. It has a logP value of 2.9. Side-effects on Anidulafungin include stomach pain, diarrhea, dizziness, constipation and vomiting.

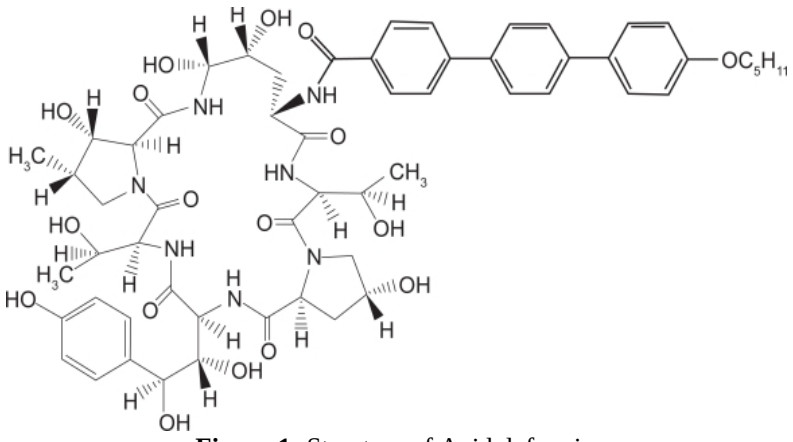


Figure 1: Structure of Anidulafungin

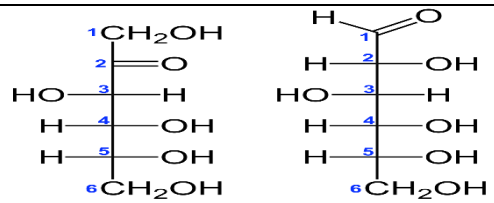
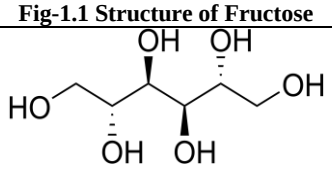
S.NO	Chemical Name	IUPAC	Chemical Structure
1.	Fructose	(3*S*,4*R*,5*R*)-2-(hydroxymethyl)oxane-2,3,4,5-tetrol	
2.	Mannitol	(2*R*,3*R*,4*R*,5*R*)-hexane-1,2,3,4,5,6-hexol	

Fig-1.1 Structure of Fructose

Fig 1.2 Structure of Mannitol

As per literature survey a few analyticalmethod have been reported for the determination of Anidulafungin in pure drug, pharmaceutical dosage form and in biological samples using Liquid Chromatography. Literature survey revealed that few analytical techniques and methods have been described for sugars analysis from different matrices. High performance liquid chromatography coupled with refractive index detection (HPLC–RI) is the most popular method used for analyses of mono-, di- and polysaccharides from concentrated carbohydrate syrups. Thus, the aim of this study was to develop, optimize and validate a simple and reproducible HPLC–RI method for the simultaneous qualitative and quantitative determination of fructose and mannitol in Anidulafungin

2. Experimental Part

Chemicals: All glassware used are made of Borosilicate, Chemicals like Methanol, Acetonitrile are manufactured by JTBaker and distilled water is used. Fructose standard (99.90 potency) and Mannitol (99.10 potency) provided by the Aurobindo Pharma Ltd, RC-I, Hyderabad.

Instrumentation: HPLC make by Waters Alliance, model e2695 with RI Detectors along with operating software Empower3 Advanced. Weighingbalance made by Sartorius model types BSA2245-CW. Sonicator made by Enertech model type SE60US. Hotair oven made by Memmert model no UF55. Refrigerator made by Samsung model Luxe black,2022.

Methodology:

- **Diluent:** Water was used as the diluent.
- **Standard Solution Preparation:** Accurately weighed 80mg of fructose and 66mg of mannitol were transferred to 10mL flasks separately and 5mL of diluents was added to the flask and sonicated for 10min.Volume was made upto mark with diluentand labelled as standard stock solutions (8mg/mL of Fructose and 6.6mg/mL of mannitol).
- **Working Standard Solution Preparation:** 2mL from each stock solution was pipetted out and taken into a 10mL volumetric flask and volume was made upto the mark with diluent.
- **Sample stock preparation:** Take marketed Anidulafungin injection. Accurately weighed 80mg of fructose and 6mg of mannitol were transferred to 10mL flasks separately and 5mL of diluents was added to the flask and sonicated for 10min.Volume was made upto mark with diluent and labelled as standard stock solution (8mg/mL of Fructose and 6.6mg/mL of mannitol).
- **Sample working standard preparation:** 2mL from each stock solution was pipetted out and taken into a 10mL volumetric flask and volume was made upto the mark with diluent

Method optimization

The separation was performed on Alltimo amino (250*4.6mm),5µm using mobile phases, water: Acetonitrile (30:70)%v/v, both the peaks were eluted but resolution between two peaks observed was not satisfactory. After several trails the column was changes to ShodexAshipack amino (250*4.6mm),5µm. The trail was run by using the mobile phase water and Methanol: Acetonitrile (20:80)% v/v. There is clear separation between two peaks resolution is 3.28. As flowrate is 0.6ml/min pressure is low 330psi. The flow rate was increased to 0.8ml/min and pressure is increased from 330psi to 440psi to achieve good chromatogram.

3. Results

Method validation Parameters as per ICH guidelines:

The method was validated for parameters like system suitability, specificity, Linearity, accuracy, precision, LOD, LOQ and robustness.

1) The system suitability

The system suitability parameters were determined by preparing standard solution of fructose and mannitol and the solution were injected six times and the parameters like peak tailing, resolution and plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%

Table 1: System suitability parameters of Fructose and Mannitol

Standard Solution injection	FRUCTOSE			MANNITOL		
Sl.no	Area	USP Plate count	Tailing	Area	USP Plate count	Tailing
1	7017618	3397	1.09	30122770	3207	1.00
2	6966346	3433	1.02	30417809	3137	0.99
3	6837164	3499	1.04	30085595	3226	0.99
4	6836904	3453	1.09	30019565	3220	0.98
5	6802828	3550	1.04	30079809	3274	0.98
6	6854942	3494	1.05	30333041	3263	0.98
Mean	6885967			30176431		
%RSD	0.2			0.52		

2) Specificity

Specificity of the method was determined by injecting blank and placebo to check whether peaks in the blank and placebo are eluting with drugs peaks. So this method was considered to be specific.

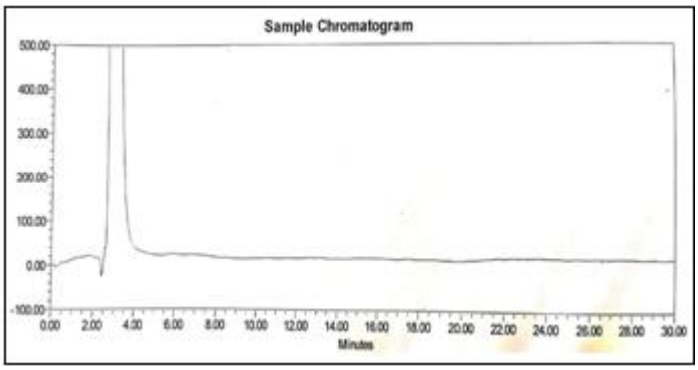


Figure 2: Chromatograms of Blank and placebo

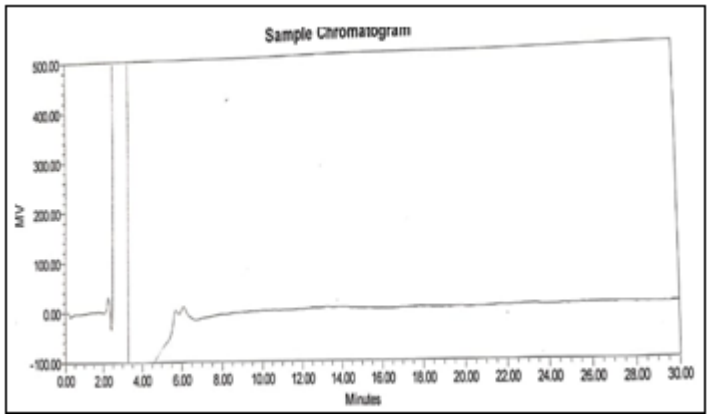


Figure 3: Chromatogram

Acceptance criteria:

No interference peaks should be found at retention time of Fructose and Mannitol from blank and placebo in this method.

3) Linearity

Injected each level into the chromatographic system and measured the peak area. Plotted a graph of peak area versus concentration (concentration on x-axis and peak area on y-axis) and calculated the R^2.The linearity was determined by injecting the LOQ, 25%,50%,75%,100%,130% of spiked solutions.

Table 2: Linearity Data of Fructose and Mannitol

Fructose			Mannitol		
% of linearity sol to test conc.	Concentration (µg/mL)	Area	% of linearity sol to test conc.	Concentration (µg/mL)	Area
25.00	1634.75	1525332	25.00	398.40	7302506
50.00	3269.50	3263281	50.00	796.80	15161770
75.00	4904.26	5104442	75.00	1195.2036	22935572
100.00	6539.01	6820224	100.00	1593.60	30287866
130.00	8500.71	9022862	130.00	2071.68	39307496

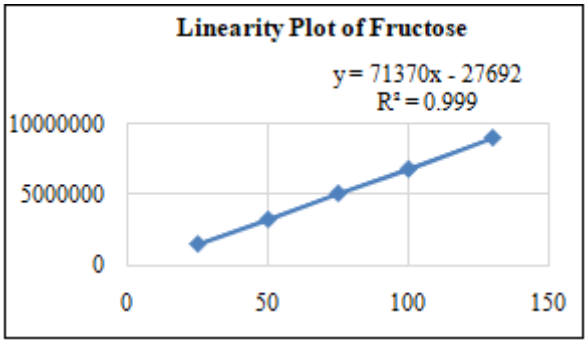


Figure 4: Linearity of Fructose

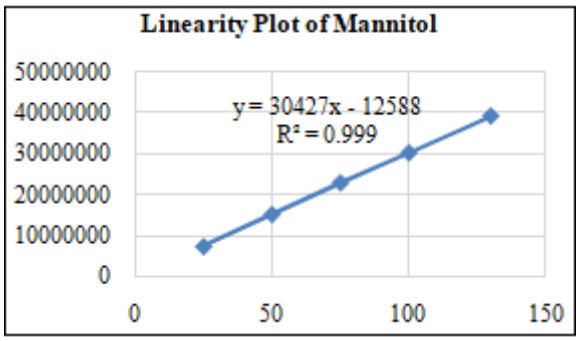


Figure 5: Linearity of Mannitol

Acceptance criteria: R^2 should be not less than 0.999.

4) Accuracy:

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. It is done by measuring the amount of pure drug recovered at three different concentrations (50%, 100% and 150%) in duplicates.

Acceptance criteria:

The mean % recovery of the fructose and mannitol in Anidulafungin at each level should be not less than 98% and not more than 105%.

Table 3: Accuracy Results for Fructose content

Recovery level	Peak area			Amt added(mg)	Amt found (mg)	% Recovery
	Inj-1	Inj-2	Avg			
50%	3482916	35469954	3476435	0.811	0.8134	100.3
100%	6916669	70001916	6959293	1.6920	1.6283	100.0
150%	10192415	10532577	10362496	2.3920	2.4246	101.4

Table.4. Accuracy results for Mannitol

Recovery level	Peak area			Amt added (mg)	Amt found (mg)	% Recovery
	Inj-1	Inj-2	Avg			
50%	15480425	15186068	15333247	3.3278	3.3345	100.2
100%	30799128	30799128	30776967	6.5584	6.6930	102.1
150%	46671974	45967427	46319701	9.8694	10.0730	102.1

Observation: The recovery results indicate that the test method has an acceptable level of accuracy. The results were found to be within the limits.

5) Precision

The method precision of the analytical method was studied by analysis of six different solutions of same concentration. The method precision is expressed as standard deviation (coefficient of variation) or relative standard deviation.

Acceptance criteria: The % Relative standard deviation of peak areas of fructose and mannitol from the six injections should be not more than 2.0 %.

Table 5: Method precision results of Fructose

S.no	PEAK AREA				Content mg/vial	Mg Found
	Volume	Inj-1	Inj-2	Avg		
Precision-1	1.00	7178288	7048629	7048629	123.7	103.9
Precision-2	1.00	7062124	7142927	7102526	124.6	104.7
Precision-3	1.00	7134213	7023649	7078931	124.2	104.4
Precision-4	1.00	6972272	7199317	7085795	124.3	104.5
Precision-5	1.00	7130279	7018187	7074233	124.1	104.3
Precision-6	1.00	6987113	7092874	7039994	123.5	103.8
				Avg	124.1	104.3
				SD	0.41	0.35
				%RSD	0.3	0.3

Table 6: Method precision results of Mannitol

S.no	PEAK AREA				Content Mg/vial	Mg Found
	Volume	Inj-1	Inj-2	Avg		
Precision-1	1.00	31816510	31589421	31702966	517.1	103.4
Precision-2	1.00	31244014	31823106	31533560	514.3	102.9
Precision-3	1.00	31500738	31557418	31529078	514.2	102.8
Precision-4	1.00	31519227	31986985	31753106	517.9	103.6
Precision-5	1.00	31560831	31709303	31635067	516.0	103.2
Precision-6	1.00	31486094	31287495	31386795	511.9	102.4
				Avg	515.2	103.1
				SD	2.18	0.44
				%RSD	0.4	0.4

Observation: From the method precision studies it was observed that %RSD of peak areas were within limits.

6) LOD & LOQ:

LOD & LOQ prediction data was also considered from the linearity experiment and calculated the method based on the standard deviation and slope was adopted.

LOD sample Preparation: From the both standard stock solution was 0.250ml pipetted out and transferred to separate 10ml flask and made up with diluent. From above solutions 0.1ml each of fructose and mannitol, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents.

LOQ sample Preparation: From the both standard stock solution was 0.250ml pipetted out and transferred to separate 10ml flask and made up with diluent. From above solutions 0.3ml each of fructose and mannitol, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents.

Table 7: LOQ&LOD Values from Linearity Data

S.NO	Name	Slope	Standard Deviation	Predicted Value of LOQ	Predicted Value of LOD
1	Fructose	71370	2690.148	0.37693	0.124387
2	Mannitol	304275	655.6071	0.09186	0.030314

7) Robustness:

Small deliberate changes in method like Flow rate, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.5ml/min), Flow plus (0.8ml/min), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected.

Table 8: Robustness Data of Fructose and Mannitol

Robustness paramarets	Fructose				Mannitol			
	RT	Area	USP plate count	USP tailing	RT	Area	USP plate count	USP tailing
Temperature increase	10.60	6832034	3755	1.01	13.25	31212625	3691	0.94
Temperature decrease	11.83	7250259	3933	1.04	15.06	31416785	4050	0.97
Flowrate increase	10.16	6514867	3733	1.01	12.83	28682489	3723	0.95
Flowrate decrease	12.43	7638400	3920	1.03	15.69	34679370	3842	0.96

Acceptance Criteria: The %RSD should be not more than 2.0

8) Solution stability:

Stability of solution is determined by analyzing at 0hrs, 6hrs and 12hrs.

Table 9: Solution Stability of Fructose

S.No	Name	RT	Area	USP plate count	USP tailing
1.	At_0hrs	11.22	6872273	3495	1.03
2.	At_6hrs	11.23	6872273	3843	1.05
3.	At_12hrs	11.21	6844590	3866	1.04

Table 10: Solution Stability of Mannitol

S.No	Name	RT	Area	USP plate count	USP tailing
1.	At_0hrs	14.18	31816510	3395	0.97
2.	At_6hrs	14.18	31109686	3829	0.96
3.	At_12hrs	14.15	31024553	3813	0.97

4. Discussion

A new HPLC method was developed for estimation of fructose and mannitol by trial and validation method i.e., by using Shodex Ashipack amino column (250*4.6mm), 5µm. The several trails were run by using the mobile phase water and Methanol: Acetonitrile (20:80) %v/v. The flow rate is 1mL/min, and we use RI detection with a detector sensitivityof512.The retention time of fructose and mannitol were found to be 10.27, 12.36 min respectively in Anidulafungin for injection Optimization of mobile phase was done based on considering factors like resolution, asymmetric factor and peak area. There is clear separation between two peaks resolution is 3.28. As flow rate is 0.8ml/min pressure is low 330psi. The flow rate was increased to 1.2ml/min and pressure is increased from 330psi to 440psi to achieve good chromatogram. Resolution between Fructose and Mannitol was found to be 4.583 which indicate good separation of both the compounds. The asymmetric factor for Fructose and Mannitol was found to be 1.420 and 1.350 respectively.

The calibration curve for Fructose and Mannitol was obtained by plotting the respective peak areas versus their concentration over the range of 12.5-75 µg/mL and 6.25-37.5 µg/mL with Regression Equation Y = 71370x-276929,correlation coefficient (r2) of 0.9999 and 0.9998 for Fructose and Mannitol respectively which indicates good correlation exist between concentration and response. Detection of limit for Fructose and Mannitol was 0.37693, 0.030314 and quantitation limit was 0.09186, 0.124387 µg/mL respectively; which suggest that the method is sensitive. The % recovery of Fructose and Mannitol was found to be in the range of 100.0-101.4% and 100.2-102.1% respectively, which shows that the developed method is accurate. The % RSD was found to be less than 2, which shows that the method was precise.

The developed RP-HPLC method for the simultaneous determination of fructose and mannitol in Anidulafungin injections is accurate, precise, and reliable. It meets all validation criteria, including specificity, linearity, and stability, thus proving its suitability for routine pharmaceutical analysis

References

[1] www.drugbank.ca/drugs/DB00362
[2] M. Pfaller, L. Boyken, R. Hollis, J. Kroeger, S. Messer, S. Tendolkar, and D. Diekema. In vitro susceptibility of clinical isolates of Aspergillus spp. to anidulafungin, caspofungin, and micafungin: a head-to-head comparison using the CLSI M38-A2 broth microdilution method. J. Clin. Microbiol. 47: 3323–3325 (2009).
[3] Pfizer Inc. Eraxis(anidulafungin) for injection. Package Insert. Pfizer, Inc. New York, NY. http://media.pfizer.com/files/products/ uspi_eraxis.pdf (2009).
[4] Christina A Sutherland, David P. Nicolau, Joseph L Kuti, 2011. “Development of an HPLC method for the determination of Anidulafungin in Human Plasma and Saline”. Journal of Chromatographic Science. Volume 49, 397 – 400. 3. Jens Martenns – Loben Hoffer, Victoria Rupprecht, Stefanie M, Bode- Boger, 2011.
[5] Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. Am J Clin Nutr. 2004;79(4):537- 43.
[6] Mannitol. Available from: https://pubchem.ncbi.nlm.nih.gov/ compound/Mannitol. [Last accessed on 10 Apr 2021] 2. Martau GA, Coman V, Vodnar DC. Recent advances in the biotechnological production of erythritol and mannitol. Crit Rev Biotechnol 2020;40:608-22.
[7] Jivraj II, Martini LG, Thomson CM. An overview of the different excipients useful for the direct compression of tablets. Pharm Sci Technol Today 2000;3:58-63
[8] J. Vazquez, Clinical therapeutics, 3(1),39(2006), https://doi.org/10.2217/14750708.3.1.39 2. Lanning Zhao, Qilong Wang, Yi Bie and Xiaoxia Lu, Journal of Pharmaceutical and Biomedical Analysis, 141, 192(2017), https://doi.org/10.1016/j.jpba.2017.04.014 3. D. Murdoch, D and G. Plosker, Drugs, 64(19), 2249(2004), https://doi.org/10.2165/00003495-200464190-00011
[9] M. Lohita, K. Swetha, P. Jaya Preethi, K. Saravanakumar and D. Naresh, Journal of Global Trends in Pharmaceutical Sciences, 5(2), 1719(2014) 5. G. Estan-Cerezo, F. Jose Rodriguez-Lucena, A. Cristina Murcia-Lopez, C. Matoses Chirivella, V. Escudero-Ortiz, A. Andujar-Mateos and A. Navarro-Ruiz. CurrentPharmaceutical Analysis, 13(3), 314(2017), https://doi.org/10.2174/1573412912666160929170016
[10] https://www.rxlist.com/eraxis-drug.html
[11] https://pubchem.ncbi.nlm.nih.gov/compound/Anidulafungin
[12] https://www.ema.europa.eu/en/documents/scientific-discussion/ecalta-epar-scientificdiscussion_en.pdf 10. A. Reboli, C. Rotstein, P. Pappas, S. Chapman, D. Kett, D. Kumar, R. Betts, M. Wible, B. Goldstein, J. Schranz, D. Krause and T. Walsh, New England Journal of Medicine, 356(24), 2472(2007), https://doi.org/10.1056/NEJMoa066906 11. S. Radha Krishna, B.M. Rao and N. Someswara Rao, Rasayan Journal of Chemistry,1(3), 467(2008).
[13] Validation on Analytical Procedures: Text and Methodology-ICH Q2 (R1),International Conference on Harmonisation, Geneva, Switzerland, (2005).