

NEW VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF CARVEDILOL IN PHARMACEUTICAL FORMULATION

BASAVESWARA RAO M.V.^{1*}, NAGENDRAKUMAR A. V. D.², YEDUKONDALU M.³, RAMAN B.V.⁴

¹Department of Chemistry, Krishna University, Machilipatnam 521001, A. P, India, ²Dept. of Chemistry, J N T University, Anantapur, A. P, India, ³Department of Chemistry, GITAM University, Visakhapatnam-530045, A. P, India, ⁴Dept. of Biotechnology, K L University, Vaddeswaram, Guntur 522502, A.P., India. Email: professorsmandava@gmail.com

Received: 8 April 2011, Revised and Accepted: 18 Sep 2011

ABSTRACT

A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Carvedilol in Bulk and Pharmaceutical tablet Formulation. Isocratic elution at a flow rate of 1.0ml/min was employed on symmetry C18 (250 mm x 4.6 mm, 5 μ m) Column at ambient temperature. The mobile phase consisted of Methanol: Acetonitrile: 1% OPA in the ratio of 80:18:2 v/v/v. The UV detection wavelength was 240nm and 20 μ l sample was injected. The retention time for Carvedilol was 2.1 min. The percentage RSD for precision and accuracy of the method was found to be 0.179%. The method was validated as per the ICH guidelines. The method was successfully applied for routine quality control analysis of pharmaceutical formulation.

Keywords: Carvedilol, RP-HPLC, UV detection, Recovery, Precise.

INTRODUCTION

Carvedilol is a third-generation nonselective chemical drug for controlling different problems of heart having chemical formula (\pm)-[3-(9H-carbazol-4-yloxy)-2-hydroxypropyl][2-(2-methoxyphenoxy) ethyl] amine (Fig-1). It has been identified as most effective and non-selective α_1 and β -adrenoreceptor (β_1 , β_2) antagonist in the treatment of systolic heart failure, possesses both reactive oxygen species (ROS) scavenging and ROS suppressive effects. It showed protective effects against daunorubicin- (DNR-) induced cardiac toxicity by reducing oxidative stress and apoptosis^{1,2}. Its antioxidant effects are attributed to its carbazole moiety. Carvedilol is taken twice daily because it is extensively metabolized and therefore loses its effectiveness due to a short half-life³. Norepinephrine stimulates the nerves that control the muscles of the heart by binding to the α_1 , β_1 - and β_2 -adrenergic receptors. Carvedilol blocks the binding to those receptors^{2,4}, which slows the heart rhythm and reduces the force of the heart's pumping. This lowers blood pressure and reduces heart failure. Relative to other beta blockers, Carvedilol has minimal inverse agonist activity⁵. Therefore, its concentration plays a decisive role in controlling the heart failure. Variation in its concentration should be measured for proper prescription of this medicine both in patients' blood and drug formulations.

Several analytical methods have been reported for the determination of Carvedilol in pure drug, pharmaceutical dosage forms and in biological samples using spectrophotometer liquid chromatography, electro kinetic chromatography high performance thin layer chromatography⁶. Pattana *et al.*, (2010)⁷ has developed that Chromatographic separation was achieved on a Alltima C18 (250 mm 4.6 mm) column using a mobile phase containing 0.01 M Na₂HPO₄ in water and acetonitrile (30:70 v/v) adjusted to pH 3.0 by ortho phosphoric acid at a flow rate of 1.0 ml/min and employing fluorescence detection with 300- nm excitation and 343-nm emission wavelengths. Patel *et al.*, (2006)⁸ emphasized that the high performance liquid chromatography method, Lichrospher 100 C-18, 5 μ m column consisting of 200 \times 4.6 mm i.d., in isocratic mode, with mobile phase containing 50mM KH₂PO₄ buffer (pH 3.0 \pm 0.1): acetonitrile: methanol (60:50:10 v/v/v) was used. The flow rate was 1.0 ml/min and effluent was monitored at 242 nm. The retention time was 4.56 \pm 0.03 min. The mobile phase consisting of ethylacetate: toluene: methanol (1:4:3.5 v/v/v). The detection of spot was carried out at 242 nm. The limit of detection and limit of quantification for high performance liquid chromatography were found to be 0.2 and 0.85 μ g/ml, respectively, and for high performance thin layer chromatography, 10 and 35ng/spot, respectively. Eric *et al.*, (2004)⁹ developed that S-Carvedilol and R-

Carvedilol are extracted from human plasma by protein precipitation using acetonitrile containing racemic [²H₅]-Carvedilol as an internal standard. The maximum within-run precision observed in a three run quality control was 8.2% for S-Carvedilol and 6.7% for R-Carvedilol, respectively. The maximum percentage bias observed at all quality control sample concentrations was 9.4% for S-Carvedilol and 11.6% for R-Carvedilol, respectively.

Galanopoulou *et al.*, (2008)¹⁰ emphasized that the separation was achieved with an X-terra C₁₈ column, using acetonitrile-phosphate buffer pH 2.5 as mobile phase. The unknown peak of degradation product was attributed to a new compound generated from the interaction of CV molecule and polyvinyl pyrrolidone (PVP) in the presence of water molecules. Peccinini *et al.*, (2008)¹¹ developed that the quantitation limit was 0.25 ng/ml for S(-)-Carvedilol in plasma and 0.5 ng/ml for R(+)-Carvedilol in plasma and for both enantiomers in urine. The data obtained demonstrated highest plasma levels for the R (+)-Carvedilol (AUC(SS) (0-12) 75.64 vs 37.29 ng/ml). The enantiomeric ratio R (+)/ S(-) was 2.03 for plasma and 1.49 for urine (Ae(0-12) 17.4 vs 11.7 microg). Ptacek *et al.*, (2003)¹² has described that the method is based on protein precipitation with methanol, concentration of the supernatant by evaporation and reversed-phase chromatography with fluorimetric detection. Only 250 μ l of plasma used for sample preparation, the limit of quantization 1.3ng/ml was achieved. Saito *et al.*, (2006)¹³ has described the mobile phase was hexane: isopropanol: diethylamine (78:22:1, v/v). The excitation and emission wavelengths were set at 284 and 343 nm, respectively. The limits of quantification for the S(-) and R(+)-Carvedilol enantiomers in plasma and blood were both 0.5 ng/ml. Intra- and inter-day variations were less than 5.9%. Soo-Hwan *et al.*, (2010)¹⁴ described that Chromatography was performed on a reversed-phase (C18) column with isocratic mobile phase for 2 min. The calibration curve was linear over the range of 2 to 100 ng/ml (R₂ > 0.9998) and the lower limit of quantitation (LLOQ) was 2 ng/ml. The 90% confidence interval for the ratio of the above mentioned two parameters were within the bioequivalence limit of 0.80-1.25.

Gannu *et al.*, (2007)¹⁵ developed that the mobile phase was made of acetonitrile, 15 mM orthophosphoric acid (37: 63), and 0.25% v/v of triethylamine, with a flow rate of 1 mL/min. Serum samples containing the Carvedilol and internal standard, amitriptyline were eluted through a C8, Chromasil KR 100 5C8 column. The intraday and inter day coefficient of variation and percent error values of the assay method were less than 5%. Keeping several advantages of quantification of Carvedilol in biological and drug formulations we have developed a sensitive, accurate, simple and versatile method.

MATERIALS AND METHODS

Chemicals and reagents

The reference sample of Carvedilol (API) was obtained from Ranbaxy, Mumbai. The Formulation CASLOT (Carvedilol) was purchased from the local market. Methanol, Acetonitrile used were of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India and orthophosphoric acid is AR Grade purchased from Local market.

Instruments

Peak HPLC containing LC 20AT pump and variable wavelength programmable UV-Visible detector and Rheodyne injector was employed for investigation. The chromatographic analysis was performed on a Chromosil C18 column (250 mm×4.6 mm, 5 μ m). Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A Denwar Analytical balance was used for weighing the materials.

Preparation of Standard Solution

For analysis 100 ppm standard solution was prepared, required concentrations were obtained from 100 ppm solution by appropriate dilution.

Preparation of sample solution

The tablets of Carvedilol (CASLOT – 12.5 mg) were crushed to give finely powdered material. From the Powder prepared a 50 ppm solution with mobile phase and then filtered through Ultipor N₆₆ Nylon 6, 6 membrane sample filter paper.

Chromatographic Conditions:

Experiment was run with Methanol: Acetonitrile: 1% OPA 80:18:2 v/v/v mobile phase at pH 6.2 using Chromosil C18 analytical column (250 mm × 4.6 mm, 5 μ m). 20 μ l of sample is injected at ambient temperature for 6 min runtime with a flow rate of 1.0ml/min and effluents were identified at 240nm. The sample retention time is 2.1Min.

Method Development

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant¹⁶⁻²².

Wavelength Detection

The spectrum of 10ppm solution of the Carvedilol in methanol was recorded separately on UV spectrophotometer. The peak of maximum absorbance wavelength was observed. The spectra of Carvedilol were showed maximum absorbance at 240nm.

Validation of the proposed method

The proposed method was validated as per ICH guidelines²⁴⁻³¹.

Linearity

Linearity was performed by preparing mixed standard solutions of Carvedilol at different concentration levels including working concentration mentioned in experimental condition i.e. 50ppm. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. The response was read at 240nm and the corresponding chromatograms were recorded. The regressions of the plots were computed by least square regression method. Linearity results were presented in Table-2 and Fig-3.

Precision

To study the intraday precision, six replicate standard solutions (70ppm) of Carvedilol were injected. The percent relative standard deviation (% RSD) was found to be 0.179. To study the interday precision, six replicate standard solutions (70ppm) of Carvedilol was injected on third day of sample preparation. The percent relative standard deviation (% RSD) was found to be 0.177, which are well within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table-3 & 4.

Specificity

The specificity of method was performed by comparing the chromatograms of blank, standard and sample (Prepared from Formulation). It was found that there is no interference due to recipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The specificity results are shown in Table-1.

Accuracy

A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level of 40ppm. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and % RSD was calculated and results are presented in Table-5. Satisfactory recoveries ranging from 99.0 to 102.0 were obtained by the proposed method. This indicates that the proposed method was accurate.

Robustness

The robustness study was performed by slight modification in flow rate of Mobile phase, pH of the buffer and composition of the mobile phase. Carvedilol at 60ppm concentration was analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature. The results of robustness study are shown in Table-6.

System suitability

System suitability was studied under each validation parameters by injecting six replicates of the standard solution (70ppm). The results obtained were within acceptable limits (Tailing factor ≤ 2 and Theoretical plate's ≥ 2000) and are represented in Table-7. Thus, the system meets suitable criteria.

Limit of detection and Limit of quantification

Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined as the lowest Concentration that can be quantified reliably with a specified level of accuracy and Precision. For this sample was dissolved by using Mobile Phase and injected until peak was disappeared. After 0.3ppm dilution, Peak was not clearly observed. So it confirms that 0.3ppm is limit of Detection and 0.99ppm dilution is Limit of Quantification. For this study six replicates of the analyte at lowest concentration were Measured and quantified. The LOD and LOQ of Carvedilol are given in Table-8.

Formulation

For assay Carvedilol (CASLOT – 12.5 mg) 20 tablets were weigh and calculate the average weight. Accurately weigh and transfer the sample equivalent to 10mg of Carvedilol in to a 10ml volumetric flask. Add diluent and sonicate to dissolve it completely and make volume up to the mark with diluents. Mix well and filter through 0.45 μ m filter. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to mark with diluents and finally 50 ppm were prepared. Mix well and filter through 0.45 μ m filter. An aliquot of this solution was injected into HPLC system. Peak area of Carvedilol was measured for the determination.

RESULT AND DISCUSSION

The system suitability method acceptance criteria set in each validation run were: capacity factor >2.0 , tailing factor $=2.0$ and theoretical plates >2000 . In all cases, the relative standard deviation (R.S.D) for the analyte peak area for two consecutive injections was $< 2.0\%$. A chromatogram obtained from reference substance solution is presented in Fig-2. The results of optimization studies showed that the mixture of Methanol, Acetonitrile in 80:15 proportions and C18 column give satisfactory results. The pH of mobile phase was adjusted to 4.5 with o-phosphoric acid.

The calibration curve for Carvedilol was obtained by plotting the peak area of Area versus concentration of Carvedilol over the range of 50-100ppm, and it was found to be linear with $r = 0.999$. The intraday and inter day precision study of Carvedilol was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days (freshly prepared) for 3 different concentrations of Carvedilol (60, 80, 100ppm). The standard deviation (Intraday-0.179, Interday-0.177), and coefficient of variation was calculated and they are within the acceptance limit.

Accuracy of the method was evaluated by the determination of recovery of Carvedilol in duplicated at three levels such as 50 %, 100 % and 150 % of the method concentration. Results of recovery studies are shown range 99.00-101.45%. The mean recovery data

obtained for each level as well as for all levels combined (Table-5) were within 2.0% of the label claim for the active substance with an R.S.D. < 2.0%, which satisfied the acceptance criteria set for the study. Percentage of recovery (8.6%) from the sample shows that the method is free from any interference because of recipients. The result obtain by proposed method were in good agreement with the label claim. The additives and recipients present in the tablet did not interference. The value of standard deviation and percentage of RSD values were satisfactorily low that indicate accuracy and reproducibility of this method. No interfering peaks were observed at the elution times of Carvedilol. The method is accurate, reproducible and specific. The retention time and in-turn run time was very short, hence required less mobile phase for the method, making it more economical and rapid.

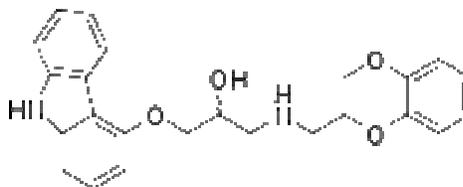


Fig. 1: Structure of Carvedilol

HPLC Report

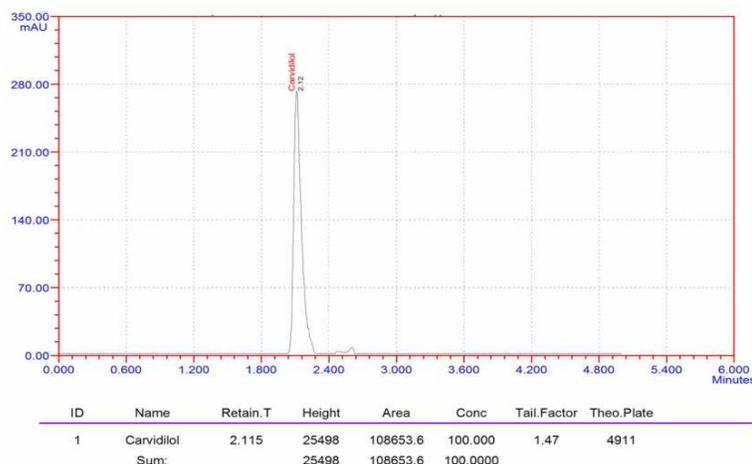


Fig. 2: A typical chromatogram of Carvedilol pure drug

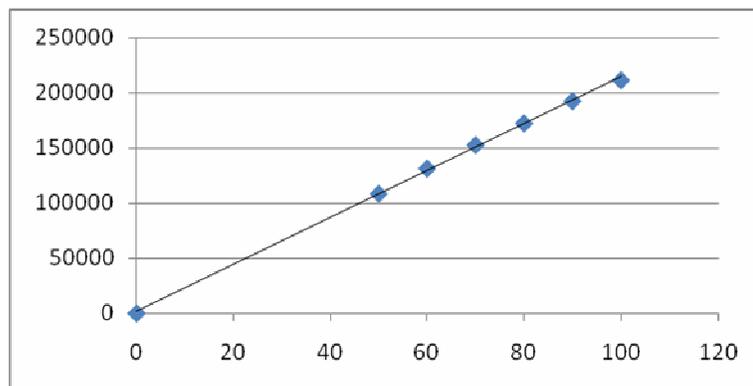


Fig. 3: Linearity Graph of Carvedilol

On X axis concentration of sample, On Y axis peak area response

Table 1: Specificity study of Carvedilol

Name of the solution	Retention Time in Min
Blank	No peaks
Carvedilol(Standard)	2.1
Carvedilol (Sample)	2.2

Table 2: Linearity study on Carvedilol

Level	Concentration of Carvedilol (ppm)	Mean peak area
Level -1	50	108653.6
Level -2	60	131546.1
Level -3	70	152872.5
Level -4	80	172604.7
Level -5	90	192851.6
Level-6	100	211735.2
Range: 50-100ppm	Slope	3871.014
	Intercept	1741.95
	Correlation coefficient	0.999

Table 3: Interday Precession of Carvedilol

Sample	Concentration (ppm)	Injection No.	Peaks area	R.S.D. (Acceptance criteria ≤2.0%)
Carvedilol	70	1	152872.5	0.179
		2	152247	
		3	152209	
		4	152247	
		5	152180	
		6	152157	

Table 4: Intraday Precession of Carvedilol

Sample	Concentration (ppm)	Injection	Peaks area	R.S.D (Acceptance criteria ≤ 2.0%)
Carvedilol	70	1	152805.2	0.177
		2	152923.1	
		3	152872.9	
		4	152608	
		5	152180	
		6	152675.6	

Table 5: Percentage of R.S.D of Carvedilol

Level	Amount of Carvedilol spiked (ppm)	Amount of Carvedilol recovered(ppm)	% Recovery	%RSD
50 %	60	59.8	99.66	0.255
	60	59.9	99.83	
	60	59.6	99.33	
100%	80	79.7	99.62	0.317
	80	80.1	100.1	
	80	79.6	99.5	
150%	100	99.8	99.8	0.180
	100	99.7	99.7	
	100	100.05	100.05	
			Mean % of recovery = 99.73	Mean RSD = 0.250

Table 6: Results of Robustness of Carvedilol

Conditions	Mean area	% Assay	% Difference
Unaltered	131546.1	100.0	0.0
Flow rate at 0.8 mL/min	130856.3	99.47	0.53
Flow rate at 1.2mL/min	131205.9	99.74	0.26
Mobile phase			
MEOH: Water	131252.6	99.77	0.33
75% 25%	130992.8	99.57	0.43
85% 15%			
pH of mobile phase at 7.3	131850.6	100.2	0.2
pH of mobile phase at 7.7	132057.6	100.3	0.3

Table 7: System Suitability parameters of Carvedilol

Parameter	Tailing factor	Theoretical plates
Specificity study	1.47	4911.49
Linearity study	1.19	3050.96
Precision study	1.21	3057.57

Table 8: LOD and LOQ of Carvedilol

Parameter	Measured volume
Limit of Quantification	0.99ppm
Limit of Detection	0.3ppm

CONCLUSION

The Proposed method is suitable technique for the reliable analysis of commercial formulation containing of Carvedilol. This method can be used for routine quality control analysis of pharmaceutical formulations.

ACKNOWLEDGEMENT

Authors thank to the management of Krishna University, Machilipatnam for providing necessary facilities to carry out this work.

REFERENCES

- Arozal W, Sari FR, Watanabe K, Arumugam S, Veeraveedu PT, Ma M, Thandavarayan RA, Sukumaran V, Lakshmanan AP, Kobayashi Y, Mito S, Soetikno V, Suzuki K. Carvedilol-Afforded Protection against Daunorubicin-Induced Cardiomyopathic Rats In Vivo: Effects on Cardiac Fibrosis and Hypertrophy. *ISRN Pharmacol*, 2011; 430549.
- Othman AA, Tenero DM, Boyle DA, Eddington ND, Fossler MJ. Population pharmacokinetics of S (-)-Carvedilol in healthy volunteers after administration of the immediate-release (IR) and the new controlled-release (CR) dosage forms of the racemate. *AAPS J*, 2007; 9 (2): E208-18.
- Hashemzadeh M, Movahed MR, Russu WA, Soroush L, Hill DN. Novel design and synthesis of modified structure of carvedilol. *Recent Pat Cardiovasc Drug Discov*. 2011; 6(3):175-9.
- Stafylas PC, Sarafidis PA. Carvedilol in hypertension treatment. *Vasc Health Risk Manag*, 2008; 4 (1): 23-30.
- Vanderhoff BT, Ruppel HM, Amsterdam PB. Carvedilol: The new role of beta blockers in congestive heart failure. *Am Fam Physician*, 1998; 58(7):1627-34.
- Suddhasattya D, Dhiraj Kumar SA, Sreenivas D, Sandeep AC. Analytical method development & validation of carvedilol by HPLC in bulk and dosage form. *Journal of Pharmacy Research*, 2010; 3(12): 3075-3077.
- Pattana S, Somsak K, Sakawrat T. Development of Carvedilol assay in tablet dosage form using HPLC with fluorescence detection. *Maejo Int J Sci Technol*, 2010; 4(1): 8-19.
- Patel LJ, Suhagia BN, Shah PB, Shah RR. RP-HPLC and HPTLC methods for the estimation of Carvedilol in bulk drug and pharmaceutical formulations. *Ind J pharma sci*, 2006; 68(6): 790-793.
- Eric Y, Sherry W, John K, Matthew JC. Stereoselective analysis of Carvedilol in human plasma using HPLC/MS/MS after chiral derivatization. *J Pharm Biom Anal*, 2004; 36(3- 15): 609-615.
- Galanopoulou O, Rozou S, Antoniadou-Vyza E. HPLC analysis, isolation and identification of a new degradation product in Carvedilol tablets. *J Pharm Biom Anal*, 2008; 48(1- 10): 70-77.
- Peccinini RG, Ximenes VF, Cesarino EJ, Lanchote VL. Stereoselective analysis of Carvedilol in human plasma and urine using HPLC after chiral derivatization. *Biopharm Drug Dispos*, 2008; 29(5): 280-8.
- Ptacek P, Macek J, Klíma J. Liquid Chromatographic Determination of Carvedilol in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*, 2003; 789 (2): 405-410.
- Saito M, Kawana J, Ohno T, Kaneko M, Mihara K, Hanada K, Sugita R, Okada N, Oosato S, Nagayama M, Sumiyoshi T, Ogata H. Enantioselective and highly sensitive determination of Carvedilol in human plasma and whole blood after administration of the racemate using normal-phase high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*, 2006; 843(1): 73-77.
- Soo-Hwan K, Sang Hun L, Hye Jung L. Rapid and Sensitive Carvedilol Assay in Human Plasma Using a High-Performance Liquid Chromatography with Mass/Mass Spectrometer Detection Employed for a Bioequivalence Study. *Am J Analy Chem*, 2010; 1: 135-14.
- Gannu R, Yamsani VV, Rao YM. New RP-HPLC method with UV-detection for the determination of Carvedilol in human Serum. *J Liq Chromatogr Relat Technol*, 2007; 30(12): 1677-1685.
- Q2A: Text on Validation of Analytical Procedures. In International Conference on Harmonization, Federal Register 1995; 60(40): 11260-11262.
- Q2B: Validation of Analytical Procedures: Methodology, Availability. In International Conference on Harmonization. Federal Register 1997; 62(96): 27463-27467.
- FDA: Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation; Availability. Federal Register (Notices), 2000; 65(169): 52776-52777.
- Vijay Kumar B, Pratap Kumar K, Suresh K, Saheena A, Srikanth P, Suneetha Y. Development and validation of RP-HPLC method for determination of Raloxifene Hydrochloride from pharmaceutical preparation. *J Chem Pharm Res*, 2011; 3(3):784-791 [www.fda.gov/cder/guidance/cmc3.pdf].
- USP 25-NF 20, Validation of Compendial Methods Section (1225) (United States Pharmacopeal Convention, Rockville, Maryland, USA, 2002) p 2256.
- Shahir GA. Validation of HPLC Chromatography Methods for Pharmaceutical Analysis. Understanding the Differences and Similarities between Validation Requirements of FDA, the US Pharmacopeia and the ICH. *J Chromatogr A*, 2003; 987(1-2): 57-66.
- Wood CE. Medicare Program; Changes to the Hospital Outpatient Prospective. *Med J Aust*, 1996; 165: 510-514.
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: definitions and terminology, Geneva (1996) adopted by Ravichandran V, Shalini S, Sundram KM, Harish R. Validation of analytical methods - strategies & importance. *Int J Pharmacy and Pharm Sci*, 2003; 2(3): 18-22.
- U.S. FDA, Title 21 of the U.S. Code of Federal Regulations: 21 CFR 211—Current good manufacturing practice for finished pharmaceuticals. [http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm].
- U.S. FDA - Guidance for Industry (draft) Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls and Documentation, 2000 [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122858.pdf].
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories, 2005 [http://www.a2la.org/requirements/req17025.pdf].
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for

- Human Use, Validation of analytical procedures: Methodology, adopted in 1996, Geneva adopted by Peinado A, Hammond J, Scott A. Development, validation and transfer of a near infrared method to determine in-line the end point of a fluidised drying process for commercial production batches of an approved oral solid dose pharmaceutical product. *J Pharm Biomed Anal.* 2011, 5; 54(1):13-20.
28. U.S. EPA, Guidance for methods development and methods validation for the Resource Conservation and Recovery Act (RCRA) Program, Washington, D.C. (1995). [<http://www.epa.gov/sw-846/pdfs/methdev.pdf>].
 29. General Chapter 1225, Validation of compendial methods, United States Pharmacopeia 30, National Formulary 25, Rockville, Md., USA, The United States Pharmacopeial Convention, Inc., (2007) adopted by Ronnie DP, JoAnn S. Medicare Part D: Selected Issues for Plan Sponsors, Pharmacists, and Beneficiaries in 2008. *J Manag Care Pharm*, 2008; 14(1): 50-60.
 30. U.S. FDA - Guidance for Industry, Bioanalytical Method Validation adopted by Shen G, Hong JL, Kong AN. Development and validation of an HPLC method for the determination of dibenzoylmethane in rat plasma and its application to the pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci*, 2007; 852(1-2): 56-61.
 31. Hokanson GC. A life cycle approach to the validation of analytical methods during pharmaceutical product development, Part I: The initial validation process. *Pharm. Tech.* 1994, pp. 118-130.