Research Article

Development and Validation of HPTLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and Cilnidipine in Combined Pharmaceutical Tablet Dosage Form

D. M. Shah, D. B. Doshi*

Department of Quality Assurance, L. M. College of Pharmacy, Ahmedabad-380009, India.

ABSTRACT

Nebivolol hydrochloride and Cilnidipine is used for the treatment of hypertension. A simple, accurate, precise and sensitive HPTLC method has been developed and validated for simultaneous estimation of Nebivolol hydrochloride and Cilnidipine in combined pharmaceutical tablet dosage form. The chromatographic separation was performed on silica gel 60 F254 HPTLC plates using Toluene: Ethyl acetate: Methanol: Ammonia, (40:20:10:1, v/v/v/v) as a mobile phase. HPTLC separation of the two drug followed by densitometric measurement at 280 nm. The drugs were satisfactorily resolved with Rf values 0.39±0.02 and 0.74±0.02 for Nebivolol Hydrochloride and Cilnidipine, respectively. The method was found to be linear in range of 200-600 ng/spot and 400-1200 ng/spot for Nebivolol hydrochloride and Cilnidipine, respectively. The correlation coefficient was found to be 0.9995 and 0.9989 for Nebivolol hydrochloride and Cilnidipine, respectively. The LOD and LOQ were found to be 27.2 ng/spot and 82.6 ng/spot for Nebivolol Hydrochloride and 14.08 ng/spot and 42.67 ng/spot for Cilnidipine respectively. The mean recovery was found to be 97.8-99.3% and 97.4-99.4% for Nebivolol Hydrochloride and Cilnidipine, respectively. The intra-day and inter-day precision was found to be within limit. The proposed method has adequate specificity, sensitivity and reproducibility for quality control assay of Nebivolol hydrochloride and Cilnidipine in combined pharmaceutical tablet dosage form.

Keywords: Analytical method validation, cilnidipine, HPTLC, nebivolol hydrochloride

Received 25 April 2016 Received in revised form 24 May 2016 Accepted 26 May 2016

***Address for correspondence:**
D. B. Doshi,
Department of Quality Assurance, L. M. College of Pharmacy, Ahmedabad-380009, India.
E-mail: divyesh_81181@yahoo.com, sdhara14@yahoo.com

1. INTRODUCTION

Nebivolol hydrochloride (NBV) is a β1 receptor blocker. It is chemically (1RS,1'RS)-1,1'-[(2RS,2'SR)-bis(6-flurochroman-2-yl)]-2,2'-iminodiethanolhydrochloride (**Figure 1**) [1,2]. Cilnidipine (CLD) is a fourth generation Ca^{2+} channel blocker. It is chemically (1,4 -Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-methoxyethyl (2*E*)-3-phenyl-2-propenyl ester) (**Figure 2**) [3,4]. NBV and CLD are used alone in treatment of hypertension but when they are given in combination synergistic action obtained and hence dose is reduced. Literature survey revealed that various analytical method like UV, HPLC, HPTLC, LC-MS and LC/MS-MS methods have been reported for estimation of NBV and CLD alone in bulk drug, pharmaceutical formulation and biological fluid [5-33]. Only two methods UV and HPLC have been reported for the estimation of NBV and CLD in combined pharmaceutical dosage forms [34, 35]. However, HPTLC method has not been reported for the simultaneous determination of both the drugs in combined tablet dosage form. Hence, the aim of the proposed work was to develop and validate simple, accurate, precise and sensitive HPTLC method for the simultaneous estimation of NBV and CLD in combined tablet dosage form.

2. Materials and Methods

2.1 Materials

Nebivolol hydrochloride and Cilnidipine were gifted by Pure Chem Pvt. Ltd. (Ankleshwar, Gujarat, India). All chemicals used were of analytical grade (CDH chemicals, New Delhi, India). Ln-beta 5 tablet was procured from the local market.

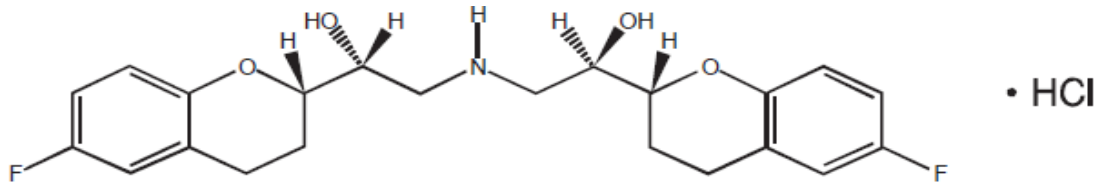

Figure 1: Structure of NBV

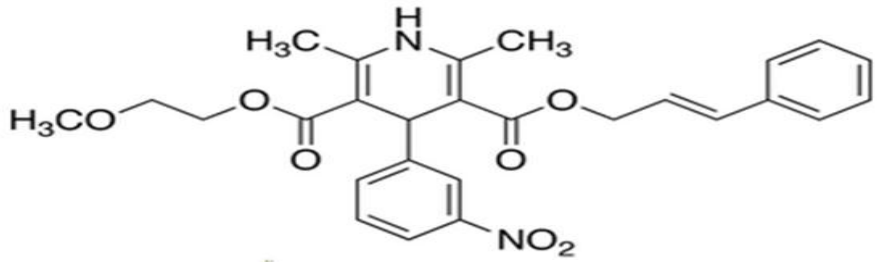

Figure 2: Structure of CLD

2.2 Instrumentation

CAMAG Linomat IV (Semiautomatic spotting device), CAMAG twin trough chamber, CAMAG TLC Scanner 3 were used. The software was CATS4. TLC Aluminum sheet pre-coated with silica gel G_{60} $F_{254,}$ layer thickness-0.2mm (Merck, Darmstadt, Germany) were used as the stationary phase.

2.3 Chromatographic Conditions

Chromatography was performed on HPTLC plates coated with 0.2 mm layers of silica gel 60 F_{254}. The plates were prewashed with methanol and activated at 110ºC for 5 min prior to chromatography. Standard and samples were spotted as 5 mm bands, 10.0 mm apart and 10 mm from the lower edge of the plate, by means of a 100µL Hamilton (Reno, Nevada, USA) micro syringe, mounted on a Linomat IV applicator; the spraying rate was 10 sec/µL. The mobile phase consisted of Toluene: Ethyl acetate: Methanol: Ammonia (40:20:10:1, v/v/v/v). Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (28±2ºC). The length of chromatogram run was 6 cm. After development the plate was dried in an oven at 110°C for 5 min. Densitometric scanning at the wavelength 280 nm was performed with a CAMAG TLC Scanner 3 equipped with win CATS4 Software Version 4.01. The slit dimensions were 3.00 mm × 0.45 mm and 100 mm/s scanning speed was employed.

2.4 Preparation of standard stock solutions

An Accurately weighed NBV (100 mg) and CLD (200 mg) were transferred to 50 mL volumetric flask and dissolved and diluted to the mark with methanol to obtain a standard stock solution of NBV (S1-2000µg/mL) and CLD (S2-4000 µg/mL).

2.5 Preparation of working standard solutions

Aliquot 5 mL of standard stock solution S1 and stock solution S2 and were transferred to 50 mL volumetric flask and diluted up to the mark with methanol. (200 µg/mL NBV and 400 µg/mL CLD). Aliquots of 1, 1.5, 2, 2.5 and 3 mL this solution were transferred to 10 mL volumetric flasks and diluted up to mark with methanol. (20-60 µg/mL NBV, 40-120 µg/mL CLD)

2.6 Preparation of test sample solutions

To determine the content of NBV and CLD simultaneously in combined tablet dosage form (label claim: 5mg Nebivolol hydrochloride and 10 mg Cilnidipine per tablet), twenty tablets were weighed, their mean weight determined and they were finely powdered. Tablet powder equivalent to 5 mg NBV and 10 mg CLD was accurately weighed and transferred to 50 mL volumetric flask containing 15 mL methanol, sonicated for 15 min and diluted to mark with methanol to obtain NBV (2000 µg/mL) and CLD (4000µg/mL).

2.7 Analytical method validation

The developed method was validated as per ICH Q2 (R1) guideline for specificity, linearity and range, precision, accuracy,

detection limit and quantitation limit parameters [36].

2.7.1 Specificity

The ability of an analytical method to unequivocally assess the analyte in the presence of other components can be demonstrated by evaluating specificity. The specificity of the HPTLC method was determined by analyzing standard drug and test sample. The spot for NBV and CLD in the sample was confirmed by comparing the R_f and spectra. The peak purity of NBV and CLD was determined by comparing the spectrum of standard drug and test sample at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

2.7.2 Linearity and range

The linearity of the developed HPTLC method was determined at the five concentration levels ranging from 200-600 ng/spot for NBV and 400-1200 ng/spot for CLD. Working standard solutions (10 µL) were spotted on HPTLC plate to obtain a final concentration range 200-600 ng/spot for NBV and 400-1200 ng/spot for CLD. The peak areas were recorded and calibration curve was constructed by plotting peak areas against concentration of drug (ng/spot)

2.7.3 Precision

The precision of the developed HPTLC method was verified by performing Intra-day, Inter-day and repeatability of sample application studies. Intra-day precision was determined by analyzing 200, 400, and 600 ng/spot of NBV and 400, 800, 1200ng/spot of CLD for three times on the same day while Inter-day precision was determined by analyzing 200, 400 and 600 ng/spot of NBV and 400, 800 and 1200 ng/spot of CLD for three consecutive days over a period of week. Repeatability was performed by spotting 10 µL working standard solution of NBV and CLD six times on HPTLC plate. Percentage relative standard deviation (%RSD) was calculated for intra-day, inter-day and repeatability studies.

2.7.4 Limit of detection (LOD)

It is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified under the stated analytical conditions. It was calculated by using following formula.

$LOD = (3.3 \times \sigma) / S$

σ = Standard deviation of the Y intercept

S = Slope of the calibration curve

2.7.5 Limit of quantitation

It is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental condition. It was calculated by using following formula.

$LOQ = (10 \times \sigma) / S$

σ = Standard deviation of the Y intercept

S = Slope of the calibration curve

2.7.6 Accuracy

To examine the accuracy of the developed HPTLC method, recovery studies were carried out by standard addition method at three different concentration levels (80, 100 and 120%) in triplicate by spiking standard NBV and CLD solution in pre-analyzed tablet solution containing 250 ng/spot NBV and 500 ng/spot of CLD.

3. RESULTS AND DISCUSSION

3.1 Chromatographic development

The goal of the present study was to develop a rapid, precise, accurate and cost effective HPTLC method for the simultaneous estimation of NBV and CLD in combined pharmaceutical tablet formulation. The TLC procedure was optimized for simultaneous determination of NBV and CLD. The mobile phase Toluene: Ethyl acetate: Methanol: Ammonia (40:20:10:1, v/v/v/v) resulted good resolution, sharp and symmetrical peaks at R_f 0.39±0.02 for NBV and 0.74±0.02 for CLD (**Figure 3**).

3.2 Method Validation

The developed HPTLC method was validated with respect to linearity, accuracy, precision, LOD, LOQ, and specificity as per ICH guideline.

3.2.1 Specificity

The mobile phase was designed to resolve both the drugs very efficiently. The R_f values of NBV and CLD were found to be 0.39±0.02 and 0.74±0.02, respectively. The peak purity of NBV was tested by comparing the standard and sample spectrum of NBV at the peak start (S), peak apex (M) and at the peak end (E) positions. Correlation between standard and sample spectrum of NBV was found to be r(S, M) =0.996 and r(M, E)=0.999. Same procedure was followed for CLD. Correlation between

standard and sample spectrum of CLD was found to be r(S, M) = 0.997 and r(M, E) =0.995. A good correlation was obtained between the standard and sample spectra of NBV and CLD indicate specificity of the proposed HPTLC method.

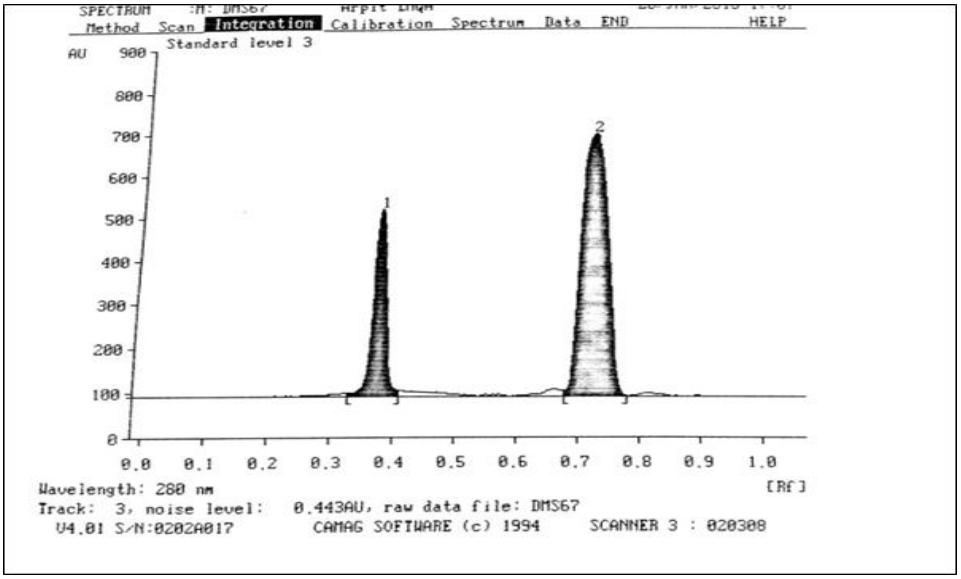

Figure 3: Densitogram of NBV (Peak 1 Rf: 0.39±0.02) and CLD (Peak 2 Rf 0.74±0.02)

3.2.2 Linearity

The method was linear in the concentration range from 200-600 ng/spot and 400-1200 ng/spot for NBV and CLD, respectively. The calibration curve was constructed by plotting concentration drug (X) versus the mean peak area of drug (Y). The correlation coefficient was found to be 0.9995 and 0.9989 for NBV and CLD, respectively. The regression equation was found as y=8.1156x + 2590.4 and y=7.2911x + 10841 for NBV and CLD, respectively. Where, y is the peak area and x is the concentration. The results showed excellent correlation between the peak area and the concentration of drug in the range tested. (**Table 1**)

Table 1: Linearity study data of NBV and CLD

Drug	Concentration (ng/spot)	Peak area (n=6) (Mean± S.D.)	% RSD
NBV	200	4200.8±46.61	1.11
	300	5077.1±35.66	0.70
	400	5782.8±68.83	1.19
	500	6650.8±29.88	0.44
	600	7471.7±40.98	0.54
CLD	400	13634.1±25.48	0.18
	600	15353.3±23.34	0.15
	800	16682.3±30.62	0.18
	1000	18196.0±31.15	0.17
	1200	19503.8±15.35	0.07

3.2.3 Precision

The %RSD for intra-day precision was found to be 0.57-0.72% and 0.04-0.19% for NBV and CLD, respectively. The % RSD for inter-day precision was found to be 0.35-1.46% and 0.09-0.23% for NBV and CLD, respectively. The %RSD for repeatability was found to be 1.87% and 0.26% for NBV and CLD, respectively. The % RSD for intra-day, inter-day and repeatability precision was found to be less than 2% indicating good precision of the developed HPTLC method. The results of precision were shown in. (**Table 2**)

Table 2: Precision study for NBV and CLD

Drug	Concentration (ng/spot)	Intra-day %RSD (n=3)	Inter-day %RSD (n=3)
NBV	200	0.57	0.82
	400	0.59	1.46
	600	0.72	0.35
CLD	400	0.19	0.21
	800	0.12	0.23
	1200	0.04	0.09

3.2.4 Limit of detection (LOD)
The LOD value for NBV and CLD were found to be 27.2 ng/spot and 14.08 ng/spot, respectively.
3.2.5 Limit of quantitation (LOQ)
The LOQ value for NBV and CLD were found to be 82.6 ng/spot for NBV and 42.7 ng/spot, respectively.

3.2.6 Accuracy
Accuracy of the proposed HPTLC method was ascertained by recovery studies and the results are expressed as % recovery. The mean recovery was found to be 97.8-99.3% and 97.4-99.4% for NBV and CLD, respectively indicates the accuracy of proposed HPTLC method. (**Table 3**)

Table 3: Recovery study of NBV and CLD

Drug	Initial amount (ng/spot)	Amount added (ng/spot)	% Recovery (n=3)
NBV	250	-	97.5±0.45
	250	200	99.3±1.52
	250	250	98.3±1.85
	250	300	97.8±2.83
CLD	500	-	97.3±0.15
	500	400	97.9±0.46
	500	500	99.4±0.61
	500	600	97.4±0.60

3.3 Analysis of marketed formulation
The proposed method was successfully applied to the analysis of marketed tablet formulation and the results obtained are given in (**Table 4**). The average drug content was found to be 96.5±0.45 and 96.3±0.15 for NBV and CLD, respectively.

Table 4: Analysis of marketed formulation

Formulation	Amount Claimed (mg)	Amount Obtained (mg) (n=3)	% Assay (n=3) Mean ±SD
Ln Beta 5	Nebivolol Hydrochloride (5 mg)	4.81± 0.58	96.5±0.45
	Cilnidipine (10 mg)	9.65± 0.34	96.3±0.15

4. CONCLUSION

Based on the results obtained, it is found that the developed HPTLC method is accurate, precise, reproducible, sensitive, specific and economical. It can become effective analytical tool for routine quality control of NBV and CLD in combined tablet dosage form.

ACKNOWLEDGEMENT

Authors are thankful to the Principal, L.M. College of Pharmacy, Ahmedabad, for providing the necessary facilities.

REFERENCES

1. Glenmark Generics (Europe) Limited, Public Assessment report, Nebivolol Tablets [online]. 2009[cited Feb]; available from: URL: www.mhra.gov.uk/home/groups/par/documents/con041409.pdf
2. Forest laboratories Canada Inc [online]. 2014 [cited May]; available from: URL: www.frx.ca/Assests/pdf/BYSTOLIC-CA_ProductMonograph_EN.pdf
3. Takahara A. Cilnidipine, A new generation Ca^{2+} channel blocker with inhibitory action on sympathetic neurotransmitter release.

Cardiovascascular Therapeutics 2009; 27(2):124-139.
4. Merck Index Laboratories, The Merck index, An Encyclopedia of Chemicals, Drugs and Biologicals, 13th edition, page no 395, 1152.
5. Walsangikar S, Ghate S, Patrakar R. Development and validation of spectrophotometric method for estimation of Nebivolol in tablet dosage forms and biological fluid. International Journal of Drug Development and Research 2010;2(3):635-642.
6. Sharma T, Patra R, Sankar DG. Development and validation of UV spectrophotometric method for determination of Nebivolol hydrochloride following ICH guidelines and study of its degradation profile. Asian Journal of Pharmaceutical and Clinical Research 2012;5(4):69-72.
7. Kamila MM, Mondal N, Ghosh LK, Gupta BK. A validated UV spectrophotometric method for estimation of Nebivolol hydrochloride in bulk and pharmaceutical formulation. Pharmazie 2007; 62 (7):486-487.
8. Shirkhedkar AA, Bugdane PM, Surana SJ. First order derivative spectrophotometric determination of Nebivolol in bulk and tablets. The Pharma Review 2008;141-143.
9. Malipatil SM, Deepthi M, Patil SK, Kiswar J. Second and third order derivative spectrophotometric estimation of Nebivolol hydrochloride in bulk and pharmaceutical dosage forms. International Journal of Pharmacy and Pharmaceutical Sciences 2011; 3(1):13-15.
10. Della P, Mathew M, Jose A, Revikumar KG. A validated UV spectrophotometric determination of an antihypertensive drug–Nebivolol from tablet formulations. International Journal of Pharmaceutical Sciences Review and Research 2010;3(2):139-141.
11. Kokilambigai KS, Lakshmi KS, Kumar A, Chandrayan G, Kumar S, Singh MK. Spectrophotometric Estimation of Cilnidipine in Bulk and Pharmaceutical Dosage Form using N-(1-Naphthyl) Ethylene Diamine Dihydrochloride. International Journal of Pharmacy and Pharmaceutical Sciences 2014;6(9):576-580.
12. Mohammed MS. Spectrophotometric Method for the Estimation of Cilnidipine in Bulk and Pharmaceutical Dosage Form. Oriental Journal of Chemistry 2013;29(1):131-134.
13. Chaudhari PP, Bhalerao AV. Method validation for spectrophotometric estimation of Cilnidipine. International Journal of Pharmacy and Pharmaceutical Sciences 2012; 4(5):96-98.
14. Indian Pharmacopoeial Commission, Indian Pharmacopoeia, Ministry of Health and Family Welfare, Government of India Ghaziabad,2014,(3),page no 2310-2312.
15. Yilmaz B. RP-HPLC method for determination of Nebivolol in pharmaceutical preparations. International Journal of Pharmaceutical Sciences Review and Research 2010;1:14-17.
16. Kokil B. Liquid chromatographic impurity profiling of Nebivolol hydrochloride from bulk drug. Der Pharma Chemica 2009;1:177-187.
17. Rajeswari KR, Sankar GG, Rao AL, Raju DB, Seshagiri Rao. RP-HPLC Method for the Estimation of Nebivolol in Bulk and Pharmaceutical Dosage Form. Asian Journal of Chemistry 2005;17(2):1259-1263.
18. Abdel FL, Abdel AL, Kosasy A, Gaeid M. Quantifiacation of Nebivolol hydrochloride in human plasma using fluroscence detection. Use in Pharmacokinetic study. Drug Discovery Therapeutics 2010;4(6):418-422.
19. Sastry BS, Srinivasulu D, Ramana H. RP-HPLC method for the analysis of Nebivolol in pharmaceutical dosage forms. Asian Journal of Pharmaceutical Research and Health Care 2009;1(1):25-33.
20. Patel LJ, Suhagia BN, Shah PB. RP-HPLC and HPTLC methods for the estimation of Nebivolol hydrochloride in tablet dosage form. Indian Journal of Pharmaceutical Sciences 2007;69:594-596.
21. Kumar MR, Basavaraj KP, Jose C, Mani T. Validated RP-HPLC method for quantitation of Nebivolol in bulk and pharmaceutical dosage form. Research Journal of Pharmacy and Technology 2010;3(4):1167-1169.
22. Sahoo G, Barik K, Kumar R. RP-HPLC method for the estimation of Nebivolol in tablet dosage form. European Journal of Chemistry 2009;6:915-919.
23. Ediga S, Goud K, Reddy KV. RP-HPLC Validation of related substances of Nebivolol in bulk and 2.5/5/10 mg tablets. International Journal of Pharmaceutical and Biological Sciences 2012;1(2):11-21.
24. Mohammed SM, Nagaraj MY. Development and validation of a Rapid Stability Indicating chromatographic determination of Cilnidipine in Bulk and Dosage form. Research Journal of Pharmacy and Technology 2013;6(3):296-299.
25. Ling-yun HE, Gao-yun HU, Yan-bin Z, Jian-hao L. Determination of Cilnidipine and its related substances by RP-HPLC. West China Journal of Pharmaceutical Sciences 2004;1:70-71.
26. Reddy TS, Devi PS. Validation of a high-performance thin-layer chromatographic

method,with densitometric detection for quantitative analysis of Nebivolol hydrochloride in tablet formulations. Journal of Planar Chromatography 2007;20:149-152.
27. Kulkarni S, Kohli S, Shinde N, Ghani S, Ratnkar I. A validated HPTLC method for determination of Nebivolol from tablets. International Journal of Pharmaceutical and Chemical Sciences 2013;2(1):350-354.
28. Karmalkar HS, Vaidya VV, Gomes NA. Determination of Cilnidipine from pharmaceutical formulation by HPTLC. Analytical Chemistry: An Indian Journal 2008;7(8):573-576.
29. Ramakrishna NV, Vishwottam KN, Koteshwara M, Manoj S, Santosh M, Varma DP. Rapid quantification of Nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2005;39:1006-1013.
30. Kyeong-Ryoon L, Yoon-Jee C, Jong-Hwa L, Dae-Duk K, Saeho C, Chang-Koo S, Suk-Jae C. Quantification of Cilnidipine in human plasma by liquid Chromatography-mass spectrometry. Journal of Liquid Chromatography & Related Technologies 2012;35:308-320.
31. Lee HW, Seo JH, Lee HS, Jeong SY, Cho YW, Lee KT. Development of a liquid chromatography/negative-ion electrospray tandem mass spectrometry assay for the determination of Cilnidipine in human plasma and its application to a bioequivalence study. Journal of chromatography B 2008;862(1):246-51.
32. Xianhua Z, Suodi Z, Rongsheng Z, Jin O, Xiaoguang L, Willy RG. Determination of Cilnidipine, a new Calcium antagonist, in human plasma using high performance liquid chromatography with tandem mass spectrometric detection. Analytica Chimica Acta 2007;1(2):142-146.
33. Zhang X, Zhai S, Zhao R, Ouyang J, Xiaoguang L, Baeyens RG. Determination of Cilnidipine, a new calcium antagonist, in human plasma using high performance liquid chromatography with tandem mass spectrometric detection. Analytica Chimica Acta 2007;1(2):142-146.
34. Thula KC, Patel DM, Maheshwari DG. Development and Validation of First Order Derivative UV Spectrophotometric Method for Simultaneous Estimation of Nebivolol and Cilnidipine in Pharmaceutical Formulation. Int J Pharm Sci Rev Res 2015; 31(1): 243-247.
35. Patel P, Patel N, Shah S. Analytical Method Development and Validation for Simultaneous Estimation of Nebivolol Hydrochloride and Cilnidipine in Combined Dosage Form. J Chem Pharm Res 2015;7(9):951-960.
36. International Conference on Harmonization (ICH), Validation of Analytical Methods: Text and Methodology Q2(R1), Geneva, 2005.