Original Article

Method development and validation of hydrochlorothiazide and Nebivolol in bulk and tablet formulation by reverse phase-high performance liquid chromatography method

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ABSTRACT

The present work describes development and validation by high-performance liquid chromatography by UV detector procedure for the analysis of hydrochlorothiazide (HCZ) and Nebivolol (NBL) in pharmaceutical mixture. Effective chromatographic separation of HCZ and NBL was achieved using a Youngline (S.K.) Gradient System UV detector. Reverse phase (GRACE) C18 column, (4.6 mm × 250 mm; 5 µm), an SP930D pump, a 20 µl injection loop, and UV 730D absorbance detector and running Autochro-3000 software. Mobile phase composed of 0.1% solution of orthophosphoric acid in water and methanol in the proportion of (40:60), respectively. The flow rate is 0.7 ml/min on detecting wavelength 282 nm. The PH of mobile phase was 3.2. The proposed high-performance liquid chromatography method was statistically validated with respect to linearity, ranges, precision, accuracy, selectivity, limit of detection, limit of quantitation and robustness. The retention time of HCZ and NBL and were found to be 4.6833 and 9.18333, respectively. All parameters were found to be within the acceptance limit. The calibration curves were linear in ranges of 25-125 and 10-50, µg/ml respectively for HCZ and NBL. The R2 of HCZ and NBL was found to be 0.999, 0.999, respectively.

Keywords: GRACE, nebivolol, reverse phase-high performance liquid chromatography, validation

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Introduction

Hydrochlorothiazide (HCZ) is 6-chloro-1, 1-dioxo-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide, molecular formula was $C_7H_8ClN_3O_4S_2$. HCZ belongs to thiazide class of diuretics. It reduces blood volume by acting on the kidneys to reduce sodium (Na+) reabsorption in the distal convoluted tubule.[1-3] The major site of action in the nephron appears on an electroneutral NaCl cotransporter by competing for the chloride site on the transporter. By impairing Na transport in the distal convoluted tubule, HCZ induces a natriuresis and concomitant water loss.[4] Thiazides increase the reabsorption of calcium in this segment in a manner unrelated to sodium transport. In addition, by other mechanisms, HCTZ is believed to lower peripheral vascular resistance.[5-7]

Nebivolol (NBL) is (6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2 hydroxyethyl] amino} ethanol, molecular formula was $C_{22}H_{25}F_2NO_4$. Table 1 NBL is unique as a beta-blocker. Unlike carvedilol, it has a nitric oxide (NO)-potentiating, vasodilatory effect. Along with labetalol, celiprolol and carvedilol, it is one of four beta blockers to cause dilation of blood vessels in addition to effects on the heart. However, recent studies question the clinical relevance of this property to NBL's efficacy.[8-13]

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Material and Methods

Chemicals and reagents

Working standards of pharmaceutical grade HCZ and NBL were obtained as generous gifts from Swapnroop Drug and Pharmaceutical. They were used without further purification. Fixed dose combination Tablet Torrent Pharma Mumbai (India) containing 10 mg NBL and 25 mg HCZ was purchased from local market. All the chemicals were of high-performance liquid chromatography (HPLC) grade, purchased from Merck Chemicals, India. Water used was double distilled and filtered through 0.45 µ filter.

Instrumentation

The analysis of the drug was carried out onYoungline (S.K.) Gradient System UV detector. Reverse phase (GRACE) C18 column, (4.6 mm × 250 mm; 5 µm), an SP930D pump, a 20 µl injection loop, and UV730D absorbance detector and running autochro-3000 software.

Preparation of standard stock and sample solution

Preparation of standard stock solution

They can accurately weighed the quantity of about 12.5 mg of HCZ and 5 mg of NBL was transferred into 10 ml volumetric flask. About 10 ml of methanol was added and sonicated to dissolve. The solution was cooled at room temperature and made up to volume with Methanol. Stock solutions of 1250 µg/ml of HCZ, 500 µg/ml of NBL obtained.

Preparation of mixed standard solution

A mixed standard solution was prepared from these stock solutions by transferring 0.1 ml of each of the stock solution to a 10 ml volumetric flask and diluting with a mobile phase to get a solution of 12.5 µg/ml and 5 µg/ml of HCZ, and NBL respectively.

Preparation of sample solution of tablet

For analysis of the tablet dosage form, 20 tablets were weighed individually, and their average weight was determined after that they were crushed to fine powders and power equivalent to weight 403 mg of tablet was weighed and transferred to 10 ml volumetric flask to form 1000 µg/ml HCZ and 1500 µg/ml NBL. Both were dissolved in HPLC grade Methanol: Acetic acid (0.1% orthophosphoric acid [OPA]) water both the solutions were shaken vigorously for 10 min and filtered through 0.45 µg nylon membrane filters. Then volume was made up to mark with Methanol: Acetic acid (0.1% OPA) water as taken and diluted to 10 ml with mobile phase to get a solution containing 10 µg/ml and 30 µg/ml, respectively. The amounts of HCZ and NBL per tablet were calculated by extrapolating the value of area from the calibration curve. The analysis procedure was repeated 5 times with tablet formulation. The result is shown in Table 2.

Results and Discussion

Method development and optimization of chromatographic conditions

The development of the method was based on the experience obtained from the HPLC method previously developed for the analysis of a mixture of analytes comprising simultaneous determination of HCZ and NBL. Experiments previously suggest the use of C18 stationary phases of (250: 4.5 mm i.d., 20 µ particle size, and 5 m). For the separation of all the two analytes in the mixture, the composition of mobile phase was varied. Parameter such as mobile phase composition of buffer was exhaustively studied so as to achieve a reasonable degree of separation of analytes. Several binary or ternary eluants were tested using different proportions of solvent, such as acetonitrile, methanol, water, and buffer at different pH conditions (Figure 1). Chromatogram obtained during method development.

Table 1: Structural formula for hydrochlorothiazide and nebivolol

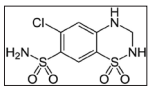
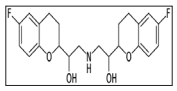
Name of drug	Hydrochlorothiazide	Nebivolol
Structural formula		

Table 2: Chromatographic conditions (HPLC) details of method development

Parameters	Conditions
HPLC	Younglin (S.K) Gradient system UV detector
Software	Autochro-3000
Column	4.6×250 mm
Particle size packing	5 µm
Stationary phase	C18 (Grace)
Mobile phase	Methanol:Acetic acid (0.1% OPA) water 60:40
Detection wavelength	282 nm
Flow rate	0.7 ml/min
Temperature	Ambient
Sample size	20 µl
pH	3.2

HPLC: High-performance liquid chromatography, OPA: Orthophosphoric acid

Initially, isocratic mode of separation was experimented and was found insufficient to resolve the mixture with good peak characters but after many trial methods developed in isocratic system. Method selected so as to achieve separation of analytes with good peak characters. The mean retention time (RT) of two analytes was and min, respectively. Peak identification was done by injecting individual analyte in developed chromatographic conditions.

Method validation

Linearity

From HCZ standard stock solution, different working standard solutions (5-25 µg/ml) were prepared in mobile phase. Likewise, from NBL standard stock solution different working standard solution (50-250 µg/ml) was prepared in mobile phase. 20 µl of the sample solution was injected into the chromatographic system using fixed volume loop injector. Chromatograms were recorded. The area for each concentration was recorded (Tables 3 and 4). The calibration curves are shown in following Figures 1-3.

Accuracy

Recovery studies were performed to validate the accuracy of the developed method. To pre-analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed. Statistical validation of recovery studies is shown in Table 5.

System suitability parameters

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of HCZ and NBL system suitability parameters were studied Table 6.

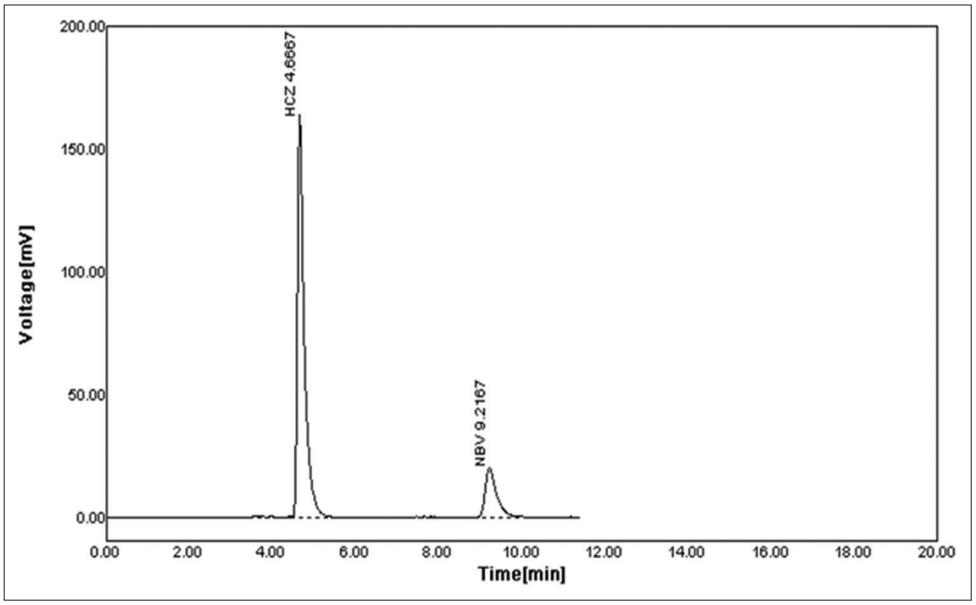


Figure 1: Chromatogram of linearity

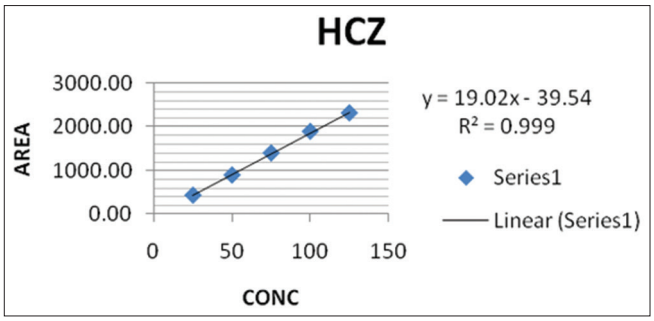


Figure 2: Calibration curve of hydrochlorothiazide

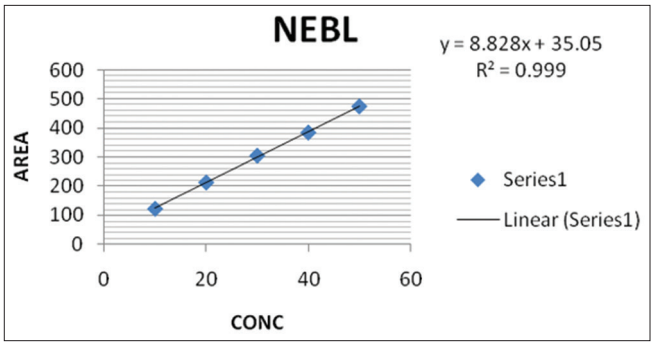


Figure 3: Calibration curve of nebivolol

Precision

The method was established by analyzing various replicates standards of HCZ and NBL. All the solution were analyzed thrice to record any intra- and inter-day variation in the result. The result obtained for interday are shown in Tables 7 and 10 the result obtained for inter-day variation are shown in the Tables 8 and 9, respectively.

Robustness

The robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate on RT and tailing factor of drug peak was studied.

The mobile phase composition was changed in ±1 ml proportion, and the flow rate was varied by ±0.7 ml min^{-1}, of the optimized

Table 3: Linearity of hydrochlorothiazide

Concentration µg/ml	Area HCZ
25	434.98
50	895.77
75	1398.50
100	1893.19
125	2314.48

HCZ: Hydrochlorothiazide

Table 4: Linearity of nebivolol

Concentration µg/ml	Area nebivolol
10	120.99
20	212.15
30	305.28
40	385.25
50	475.85

Table 5: Statistical validation of recovery studies

Level of recovery (%)	Drug	Mean % recovery	SD	% RSD
80	HCTZ	98.90	0.50	0.51
	NEBI	100.86	1.51	1.50
100	HCTZ	96.84	0.28	0.28
	NEBI	102.57	0.97	0.88
120	HCTZ	99.34	0.74	0.75
	NEBI	100.76	0.81	0.81

SD: Standard deviation, RSD: Relation standard deviation

chromatographic condition. The results of robustness studies are shown in Tables 11 and 12. System suitability parameters were also found satisfactory; hence, the analytical method would be concluded.

Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. LOD is expressed as a concentration at a specified signal to noise ratio. It may be calculated based on standard deviation of the response and slope of the curve.

LOD=3.3(SD)/S

Where, SD = Standard deviation ofY intercept

S=Slope

Limit of quantitation (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Table 6: Result of system suitability parameters

System suitability parameters	Proposed method	
	HCZ	Nebivolol
Retention time	4.7000	9.1167
Area	1844.84	378.88
Theoretical plate number	5757.1	7198.5
Tailing factor	1.2758	1.2186

HCZ: Hydrochlorothiazide

Table 7: Intra-day precision study of hydrochlorothiazide

Concentration µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
25	440.19	439.7	439.94	0.35	1.08
75	1374.14	1379.73	1376.94	1.95	0.29
125	2338.49	2367.27	2352.88	2.35	1.86

SD: Standard deviation, RSD: Relation standard deviation

Table 8: Inter-day precision study of hydrochlorothiazide

Concentration µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
25	441.19	540.7	490.94	0.26	1.23
75	1375.14	1480.73	1427.9	0.03	1.02
152	1239.50	2467.27	2403.3	0.56	1.45

SD: Standard deviation, RSD: Relation standard deviation

Table 9: Intra-day precision study of nebivolol

Concentration µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	127.9	126.28	127.09	1.15	0.90
30	307.26	308.95	308.10	1.20	0.39
50	478.31	479.95	479.13	1.16	0.24

SD: Standard deviation, RSD: Relation standard deviation

LOQ=10(SD)/S

Where, SD = Standard deviation Y intercept

S=Slope

Specificity can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample material.[14-21]

HCZ=LOD=1.30

NBL=LOD=1.13

LOQ=3.96

LOQ=3.42

Ruggedness

The ruggedness of an analytical method in which the degree of reproducibility of test result obtained by the analysis of the same sample under a variety of environmental condition that may differ but are still within the specified parameters of the assay. Result of ruggedness study was shown in Table 13-14.

Table 10: Inter-day precision study of nebivolol

Concentration µg/ml	Peak area		Mean area	SD	% RSD
	Trial 1	Trial 2			
10	128.10	125.29	126.06	1.05	0.85
30	305.25	309.95	307.6	1.19	0.35
50	479.30	480.95	480.12	1.14	0.25

SD: Standard deviation, RSD: Relation standard deviation

Table 11: Result of robustness study of hydrochlorothiazide

Parameters	Concentration	Mean±SD	% RSD
Mobile phase composition-(61+39)	75	1521.30±9.38	0.62
Mobile phase composition-(59+41)	75	1511.61±11.96	0.79
Wavelength change 281 m	75	1641.18±22.30	1.36
Wavelength change 283 nm	75	2284.28±2.74	0.73
Flow rate change (0.6 ml)	75	1197.46±3.06	0.26
Flow rate change (0.8 ml)	75	1596.89±21.20	1.33

SD: Standard deviation, RSD: Relation standard deviation

Table 12: Result of robustness study of nebivolol

Parameters	Concentration	Mean±SD	% RSD
Mobile phase composition-(61+39)	30	285.26±0.54	0.19
Mobile phase composition-(59+41)	30	282.58±2.04	0.72
Wavelength change 281 nm	30	282.63±1.76	0.62
Wavelength change 283 nm	30	254.32±0.62	0.24
Flow rate change (0.6 ml)	30	234.28±4.07	1.74
Flow rate change (0.8 ml)	30	320.63±4.48	1.40

SD: Standard deviation, RSD: Relation standard deviation

Table 13: Result of ruggedness study of hydrochlorothiazide

HCZ							
Concentration	Analyst-1	II	Mean	Amount found	% amount found	SD	RSD
75	1398.50	1499.52	1449.01	78.23	104.30	0.23	1.02

HCZ: Hydrochlorothiazide, SD: Standard deviation, RSD: Relation standard deviation

Table 14: Result of ruggedness study of nebivolol

Nebivolol							
Concentration	Analyst-1	II	Mean	Amount found	% amount found	SD	RSD
30	305.28	315.28	310.28	31.17	103.92	0.56	1.35

SD: Standard deviation, RSD: Relation standard deviation

Conclusion

Attempts were made to develop reverse phase (RP)-HPLC method for simultaneous estimation of HCZ and NBL from the tablet. For the RP-HPLC method, Younglin (S.K) isocratic system UV detector and C18 column with 250 mm × 4.6 mm i.d and 5 µm particle size. Methanol: Acetic water (0.1% OPA), (60:40) v/v, pH 3.2 was used as the mobile phase for the method. The detection wavelength was 282 nm and flow rate was 0.7 ml/min. In the developed method, the RT of HCZ and NBL sodium were found to be 3.4667 min and 5.4000 min. The developed method was validated according to the ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence, the method was found to be simple, accurate, precise, economic, and reproducible.

Hence, the proposed methods can be used for the routine quality control analysis HCZ and NBL in bulk drug as well as in formulations.

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