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A New RP-HPLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and Hydrochlorthiazide in Dosage Forms

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ABSTRACT

A new RP-HPLC method has been developed for the simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms, using ultra violet (UV) detector. Elution was carried out using a mobile phase consisting of acetonitrile and potassium dihydrogen phosphate buffer (pH 3.2 ± 0.1) in the ratio of 50:50 v/v and flow rate was set on 1.2 ml/min at 282 nm. The retention time for hydrochlorothiazide and nebivolol hydrochloride was found 3.57 and 6.66 mins, respectively. The method was found to be linear in the range of 8–32 μg/ml and 20–80 μg/ml for nebivolol hydrochloride and hydrochlorothiazide, respectively. In the linearity study, regression equation and coefficient of correlation for nebivolol hydrochloride and hydrochlorothiazide were found to be: y = 284761x + 215452, r = 0.9995; and y = 698395+105272, r = 0.9998, respectively.

Key words: HPLC, Hydrochlorthiazide, method validation, nebivolol hydrochloride

DOI: 10.4103/0975-1483.57078

INTRODUCTION

Nebivolol Hydrochloride, 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl]amino} ethanol is a white odourless powder used for the treatment of hypertension. Its mode of action is lowering blood pressure (BP) by reducing peripheral vascular resistance, and significantly increases stroke volume with preservation of cardiac output. The net hemodynamic effect of nebivolol is the result of a balance between the depressant effects of beta-blockade and an action that maintains the cardiac output.[1,2]

Hydrochlorothiazide, 6-chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide is a white or almost white odorless powder used for the treatment of high blood pressure and management of edema. Hydrochlorothiazide promotes water loss from the body (diuretics). It inhibits $Na^{(+)}Cl^{(-)}$ reabsorption from the distal convoluted tubules in the kidneys. It also causes loss of potassium and an increase in serum uric acid. It causes vasodilatation by activating calcium-activated potassium channels (large conductance) in vascular smooth muscles and inhibiting various carbonic anhydrases in vascular tissue.[3,4]

The methods found in the current literature are HPLC,[5] UV,[6] and HPTLC[7] for nebivolol hydrochloride and HPLC,[7–14] spectrophotometry,[15] diffuse reflectance spectroscopy,[16] and HPTLC[17] for hydrochlorthiazide in single as well as in combination with other drugs but

there is no new method found in the literature for the simultaneous estimation of nebivolol hydrochloride and hydrochlorthiazide.

MATERIALS AND METHODS

Shimadzu HPLC equipped with LC-10Advp pump and SPD-10Avp detector. ODS Hypersil C-18 (250×4.6 mm, 5.0 μm) Electro company were used for the development of method. Active pharmaceutical ingredients of nebivolol hydrochloride and hydrochlorthiazide batch no. 5N0024 and HTA06 are received from Cadila and Torrent Pharma, respectively. Market formulation of this combination Nebilong-H (Micro Carsyon) and Nebicard-H (Torrent) were purchase from the local market. Standards of both drugs were provided by Oasis Test House, Jaipur. All the solvents used were HPLC grade purchased from Merck ltd., Mumbai.

Chromatographic conditions were optimized by using Acetonitrile: Buffer (50:50), 1.2 ml/min flow rate and run time was kept for 12 mins. 0.30M potassium dihydrogen phosphate (4.0827 gm dissolve in 1000 ml of water) with adjusted pH 3.2 with o-phosphoric acid was used as buffer in mobile phase.

Preparation of stock solution of nebivolol hydrochloride

Nebivolol hydrochloride equivalent to 50 mg (55.14 mg taken) of nebivolol was weighed and transferred to a 50 ml volumetric flask. Approximately 40 ml of acetonitrile was added and the contents were sonicated to dissolve. The volume was made up to the mark with acetonitrile and this final dilution contained approximately 1000 μg/ml of nebivolol.

Preparation of stock solution of hydrochlorothiazide

Hydrochlorothiazide equivalent to 125 mg (126.49 mg taken) was accurately weighed and transferred to a 50 ml volumetric flask. Approximately 40 ml of acetonitrile was added and sonicated to dissolve and was made up to the mark with acetonitrile. The final dilution contained approximately 2,500 μg/ml of hydrochlorothiazide.

The λ_{max} of the two ingredients i.e., nebivolol hydrochloride and hydrochlorothiazide, were found to be 282 and 271 nm, respectively. The concentration of nebivolol in the combination tablet was less than half that of hydrochlorothiazide; hence, to obtain a better response (peak area) for the combination, the λ_{max} of the lower concentration drug i.e., nebivolol hydrochloride (282 nm) was selected for the analysis.

Preparation of synthetic mixture of nebivolol hydrochloride and hydrochlorothiazide

10 ml each of the stock solutions of nebivolol hydrochloride and hydrochlorothiazide were transferred to a 100 ml volumetric flask and was sonicated. The volume was made up to the mark with mobile phase. The resultant solution contained 250 μg/ml of hydrochlorothiazide and 100 μg/ml of nebivolol.

The peak was observed with good resolution and minimum tailing. The retention time for hydrochlorothiazide and nebivolol HCl were 3.57 and 6.66, respectively.

Here, the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drugs.

Preparation of calibration graph

4, 6, 8, 10, 12, 14, and 16 ml of the synthetic stock solution of nebivolol hydrochloride (100 g/ml) and hydrochlorothiazide (250 μg/ml) were transferred to a series of seven 50 ml volumetric flasks to prepare calibration curve. The volume in each flask was adjusted to 50 ml with mobile phase and mixed; as to obtain a final concentration in the range of approximately 20–80 μg/ml and 8–32 μg/ml of hydrochlorothiazide and nebivolol, respectively. The solutions were filtered through a 0.22 μm millipore filter and degassed under ultrasonic bath prior to use. This solution was injected into HPLC system in the triplicate. The run time was 12 mins and peak areas were measured. Calibration curve data was subjected to plot calibration graph between concentration (μg/ml) and area (mv*sec) for the mean of triplicate injection of synthetic mixture of drugs.

Regression equation and coefficient of correlation for nebivolol hydrochloride and hydrochlorothiazide were found to be: $y = 284761x + 215452$, $r = 0.9995$; and $y = 698395+105272$, $r = 0.9998$, respectively.

Validation of proposed method

Specificity

Lactose IP 60 mg, Magnesium Stearate 2 mg, Microcrystalline Cellulose IP 40 mg, Starch IP 70 mg, Talc IP 5 mg, and Titanium Dioxide 1 mg were transferred to a 100 ml volumetric flask and approximately 20 ml of methanol

was added. This solution was sonicated for 20 minutes to dissolve and the volume was made up to the mark with methanol. This solution (10 ml accurately measured) was transferred into a 50 ml volumetric flask and 10 ml of the synthetic mixture solution was added to it and volume was made up to the mark with mobile phase. The resultant solution was filtered through a 0.22 μm millipore filter and degassed under ultrasonic bath prior to use. This solution was analyzed as per the proposed method.

The comparison of the chromatograms of the synthetic mixture and the spiked drug solution revealed that there was no interference of placebo with the peaks of nebivolol hydrochloride or hydrochlorothiazide. Peak purity plots also indicated that the peaks of drugs were pure and did not have any coelution peak. No interference from placebo was observed at the retention time of the drugs. Therefore, it was concluded that the method is specific.

Linearity and range

Linearity range

In this case it is equal to 8–32 μg/ml for nebivolol hydrochloride and 16–80 μg/ml for hydrochlorothiazide.

Target range

It is range of the concentrations which is lies among the 80%, 100%, and 120% of the target concentration. In this case these are equal to 16 μg/ml, 20 μg/ml, and 24 μg/ml for nebivolol hydrochloride and 40 μg/ml, 50 μg/ml, and 60 μg/ml for hydrochlorothiazide.

Accuracy

Accuracy was assessed using a minimum of three concentration levels (nebivolol hydrochloride 8, 10, and 12 μg/ml and hydrochlorothiazide 20, 25, and 30 μg/ml) in three replicate injections. The standard solution was added in the preanalyzed tablet solution (10 μg/ml nebivolol and 25 μg/ml hydrochlorothiazide), and preanalyzed synthetic mixture (10 μg/ml nebivolol and 25 μg/ml hydrochlorothiazide) solution, respectively, in triplicate. Accuracy studies were carried out as the following procedure:

The powdered sample of the tablet (Nebilong-H) equivalent to 5 mg of nebivolol hydrochloride and 12.5 mg of hydrochlorothiazide was transferred to a 100 ml volumetric flask and approximately 90 ml of HPLC grade acetonitrile was added and sonicated to dissolve the contents. Finally the volume was made up to the mark with acetonitrile and filtered through the Whatman 41. The final solution contained about 50 μg/ml nebivolol hydrochloride and 125 μg/ml of hydrochlorothiazide. 10 ml of the above solution was diluted to 50 ml with mobile phase. The solution was filtered through a membrane filter (0.22 μm) and sonicated to degas. The dilution contained approximately 10 μg/ml nebivolol hydrochloride and 25 μg/ml of hydrochlorothiazide, and estimation of the drugs were done by proposed method.

4, 5, and 6 ml of synthetic stock solution was transferred in to three 50 ml volumetric flasks respectively, and 5 ml of synthetic standard stock solution (100 μg/ml nebivolol hydrochloride and 250 μg/ml of hydrochlorothiazide) was added in each volumetric flask and volume was made up to 50 ml with the mobile phase. These solutions were filtered through the membrane filter (0.22 μm) and sonicated to degas. The solutions were then injected into the HPLC system and chromatograms were recorded. The estimation of drugs was done by proposed method. Same procedure was followed for Nabicard-H tablet. The results of recovery studies for Nebilong-H and Nabicard-H tablets are shown in Table 1 and 2 respectively.

4, 5, and 6 ml of synthetic stock solution was transferred into three 50 ml volumetric flasks respectively, and 5 ml of synthetic standard stock solution (100 μg/ml nebivolol hydrochloride and 250 μg/ml of hydrochlorothiazide) was added in each volumetric flask and the volume was made upto 50 ml with the mobile phase. These solutions were filtered through the membrane filter (0.22 μm) and sonicated to degas. The solutions were then injected into the HPLC system and chromatograms were recorded. The estimation of drugs was done by proposed method. The result of recovery study of synthetic mixture is shown in Table 3.

The mean% recovery was found to be 99.98% and 100.38% for nebivolol hydrochloride and 101.1% and 100.06% for

Table 1: Result of Recovery study of nebivolol hydrochloride and hydrochlorothiazide in combined dosage form (Nebilong-H)

Conc. before spiking (μg/ml)	Reference Std. added* (μg/ml)	Conc. after spiking* (μg/ml)	% Recovery
Nebivolol Hydrochloride			
10.89	8.10	18.99	100.11
	10.37	21.17	99.19
	12.09	23.05	100.63
Mean ± SD	99.98 ± 0.727		
Hydrochlorothiazide			
25.08	20.05	45.11	99.90
	24.92	50.46	101.87
	30.08	55.54	101.27
Mean ± SD	101.1± 0.974		

*in triplicate

Table 2: Result of Recovery study of nebivolol hydrochloride and hydrochlorothiazide in combined dosage form (Nebicard-H)

Conc. before spiking (µg/ml)	Reference Std. added* (µg/ml)	Conc. after spiking* (µg/ml)	% Recovery
Nebivolol Hydrochloride			
10.89	8.2	18.96	99.31
10.89	10	21.22	101.58
10.89	11.76	22.62	99.86
Mean ± SD	99.98 ± 0.727		
Hydrochlorothiazide			
25.08	20.26	45.11	99.5
25.08	25.24	50.46	100.28
25.08	29.66	55.54	101.46
Mean ± SD	101.1± 0.974		

*in triplicate

Table 3: Result of Recovery study of nebivolol hydrochloride and hydrochlorothiazide in synthetic mixture

Conc. before spiking (µg/ml)	Reference Std. added* (µg/ml)	Conc. after spiking* (µg/ml)	% Recovery
Nebivolol Hydrochloride			
10.63	8.12	18.83	101.00
	10.01	20.73	100.90
	12.13	22.67	99.25
Mean ± SD	100.38 ± 0.977		
Hydrochlorothiazide			
25.13	20.08	45.06	99.30
	25.07	50.19	99.98
	30.12	55.52	100.90
Mean ± SD	100.06 ± 0.801		

*in triplicate

hydrochlorothiazide, for the tablet and synthetic mixture, respectively. The limit for mean recovery is 98–102%, and thus, the method is accurate in nature.

Precision

Injection repeatability was assessed using six determinations at 100% of the test concentration (i.e., 20 and 50 g/ml and of nebivolol hydrochloride and hydrochlorothiazide, respectively). For intraday studies, three concentrations were injected in triplicate into the HPLC system and for interday studies three concentrations (16, 20, and 24 µg/ml for nebivolol hydrochloride and 40, 50, and 60 µg/ml for hydrochlorothiazide) were injected in triplicate into the HPLC system for three days.

The repeatability study which was conducted on the solution having the concentration of approximately 50–100 µg/ml (n = 6) showed an RSD of 0.214% for nebivolol hydrochloride and 0.200% for hydrochlorothiazide. It was concluded that the analytical technique showed a good repeatability precision.

The intraday and interday precision study which were conducted, showed an RSD of 0.525% for nebivolol hydrochloride and 0.214% for hydrochlorothiazide for intra day analysis and a RSD of 0.214% for nebivolol hydrochloride and 0.104% for hydrochlorothiazide for interday analysis. Thus the data showed that the RSD was below 2% inferring that the analytical technique had a good intraday and interday precision.

Robustness

The Robustness was determined by injecting triplicate injections of standard and three-sample solutions in single at each different condition with respect to control condition. Robustness of the method was checked by varying the instrumental conditions; flow rate (±0.1 ml/min), temperature (±5°C) and change in pH of buffer (±0.2). Sample solution was injected in each condition and mean, standard deviation, and RSD were calculated.

The result obtained for the robustness study by small, deliberate changes in different chromatographic parameters indicated that the RSD of each parameter was under acceptable limit which is 2%. The results of robustness study were given in Table 4 and 5 for nabivolol and hydrochlorthiazide respectively.

System suitability parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that

Table 4: Calculation of overall RSD nebivolol hydrochloride for robustness

Assay (mg/Tablet)							
Sample S. No.	Control	Flow rate 1.3 ml/min	Flow rate 1.5 ml/min	Column oven Temp. 25°c	Column oven Temp. 35°c	pH 3.0 (–0.2)	pH 3.4 (+0.2)
1	4.88	4.94	4.83	4.85	4.83	4.88	4.75
2	4.82	4.82	4.85	4.81	4.84	4.73	4.91
3	4.87	4.84	4.84	4.88	4.88	4.91	4.82
MEAN ± SD	4.86 ± 0.03	4.87 ± 0.06	4.84 ± 0.01	4.85 ± 0.04	4.85 ± 0.03	4.84 ± 0.10	4.83 ± 0.08
RSD	0.007	0.013	0.002	0.007	0.005	0.020	0.017

Table 5: Calculation of Overall RSD hydrochlorothiazide for robustness

Assay (mg/Tablet)							
Sample S. No.	Control	Flow rate 1.3 ml/min	Flow rate 1.5 ml/min	Column oven Temp. 25°c	Column oven Temp. 35°c	pH 3.0 (–0.2)	pH 3.4 (+0.2)
1	12.39	12.37	12.39	12.41	12.42	12.39	12.43
2	12.38	12.39	12.38	12.42	12.39	12.41	12.39
3	12.42	12.41	12.42	12.41	12.41	12.41	12.41
MEAN ± SD	12.40 ± 0.028	12.39 ± 0.020	12.40 ± 0.0208	12.41 ± 0.006	12.41 ± 0.015	12.40 ± 0.011	12.41 ± 0.020
% RSD	0.168	0.161	0.168	0.047	0.123	0.093	0.161

can be evaluated as such. Table 6 shows the summary of system suitability parameters of the presented study.

Estimation of nebivolol hydrochloride and hydrochlorothiazide in tablet dosage form

Tablets of label claim of Hydrochlorthiazide and Nebivolol 12.5 and 5 mg, respectively, were used for analysis. Twenty tablets were taken, and accurately weighed (3.7908 gm for Nebilong-H and 4.0272 for Nebicard-H, respectively). The tablets were crushed to a fine powder. The powder sample equivalent to 25 mg of nebivolol hydrochloride and equivalent to 50 mg of hydrochlorothiazide (0.1895 gm for Nebilong-H and 0.2013 gm for Nebicard-H) was transferred to a 100 ml volumetric flask and approximately 80 ml of HPLC grade methanol was added and sonicated to dissolve. The volume was made up to the mark with methanol. This solution was filtered through the Whatman filter paper 41. 10 ml of this solution was diluted to 50 ml with mobile phase. The solution was filtered through a membrane filter (0.22 μm) and sonicated to degas. The solution prepared was injected in triplicate into the HPLC system and the observations were recorded. A duplicate injection of the standard solution was also injected into the HPLC system and the chromatograms were recorded. The results of the assay of Nabilong-H and Nabicard-H tablets are presented in Table 7 and 8 respectively.

RESULT

The percentage assay of nebivolol hydrochloride was found to be 99.15 0.55 and 99.06 ± 0.19%, for Nebilong-H and Nebicard-H tablets, respectively. Similarly percentage assay of hydrochlorothiazide was found to be 99.17 ± 0.24 and 98.98 ± 0.124, for Nebilong-H and Nebicard-H tablet, respectively.

Table 6: Results of system suitability parameter

Parameter	Limit	Result
Resolution	Rs > 2	3.84
Injection precision	RSD < 1% for n ≥ 5	Nebivolol HCl: RSD=0.200%, (n = 6) Hydrochlorothiazide: RSD = 0.214%, (n = 6)
Tailing factor	T ≤ 2	Nebivolol HCl: 1.24 Hydrochlorothiazide: 1.32
Theoretical plate	N > 2000	Nebivolol HCl 3658 Hydrochlorothiazide 4725
Retention time		Nebivolol HCl 3.66 min Hydrochlorothiazide 6.57 min

CONCLUSION

An RP-HPLC method has been developed for the simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms, using the UV detector. Different chromatographic conditions were used to develop the method. Elution was carried out using a mobile phase consisting of acetonitrile and potassium dihydrogen phosphate buffer (pH 3.2 ± 0.1) in the ratio of 50:50 v/v and flow rate was set on 1.2 ml/min at 282 nm. The retention time for hydrochlorothiazide and nebivolol hydrochloride was found to be 3.57 and 6.66 mins, respectively; run time was found to be 12 mins. Overall summary of validation parameters are presented in Table 9.

Table 7: Results of assay (Nebilong-H)

Peak area (μV*sec)	Labeled quantity in tablet (mg/tab)	% of Drug	Quantity found in (mg/tab)
Nebivolol Hydrochloride			
5486398	5.0	99.41	4.97
5475521		99.52	4.98
5482247		98.51	4.93
Mean SD		99.15 ± 0.55	4.96 ± 0.027
Hydrochlorothiazide			
37008007	12.5	99.36	12.42
36984451		99.11	12.38
36983117		98.88	12.36
Mean SD		99.17 ± 0.24	12.39 ± 0.03

Table 8: Results of assay (Nebicard-H)

Peak area (μV*sec)	Labeled quantity in tablet (mg/tab)	% of Drug	Quantity found in (mg/tab)
Nebivolol Hydrochloride			
5465571	5	99.22	4.96
5458332		98.85	4.94
5462210		99.11	4.96
Mean		99.06 ± 0.19	4.95 ± 0.009
Hydrochlorothiazide			
36844215	12.5	99.12	12.39
36812447		98.94	12.37
36794221		98.88	12.36
Mean SD		98.98 ± 0.124	99.06 ± 0.015

Table 9: Summary of validation parameters

Parameter	Observation	
	Hydrochlorothiazide	Nebivolol hydrochloride
Specificity	No interference was found w.r.t. excipients	
Linearity		
(Correlation coefficient r)	0.9998	0.9995
Range	70 to 130%	70 to 130%
Accuracy* (% Recovery)		
Sample	101.1%	99.98%
Mixture	100.06%	100.38%
Precision RSD***		
Repeatability (n= 6)	0.200	0.214
Intra-day (n=3)	0.214	0.525
Inter-day (days=3)	0.104	0.214
Robustness Overall RSD***		
Change in pH of mobile phase		
pH 3.0	0.093	0.020
pH 3.4	0.161	0.017
Change in temperature		
Temp 250C	0.047	0.007
Temp 350C	0.123	0.005
Change in flow rate		
1.1ml/min	0.161	0.013
1.3ml/min	0.168	0.002

*Acceptance Criteria 98-102%, **Acceptance Criteria: RSD ≤ 2%, ***Acceptance Criteria: RSD ≤ 2%

The newly developed method can be used for routine analysis as method for the simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms.

ACKNOWLEDGMENT

Oasis Test House, Jaipur, for providing facilities and reference standards of drugs.

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Source of Support: Nil, **Conflict of Interest:** None declared.