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Development and Validation for the Simultaneous Quantification of Nebivolol Hydrochloride and Hydrochlorothiazide by UV Spectroscopy, RP-HPLC and HPTLC in Tablets

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Abstract: Simultaneous quantification of nebivolol hydrochloride (NEB-H) and hydrochlorothiazide (HCT) in tablets by UV spectroscopy, RP-HPLC and HPTLC methods were developed. In UV spectrophotometric determination NEB-H and HCT was quantified by simultaneous equation method and absorbance ratio method. In simultaneous equation method absorbance measurements at 282.5 nm (λ_{max} NEB-H) and 271.5 nm (λ_{max} HCT), in absorbance ratio method absorbance measurements at 282.5 nm and 275 nm (iso absorptive point) in methanol. In RP-HPLC method, the drugs were resolved using a mobile phase of 30 mM phosphate buffer (K_2HPO_4), acetonitrile and triethylamine (50:50:0.1% v/v) with pH 5.5 using orthophosphoric acid on a C_{18}-ODS- Phenomenex (5 µm, 250 mm x 4.6 mm) column in isocratic mode, Atorvastatin (ATR) used as a internal standard. The retention time of HCT, NEB-H and ATR was 3.31, 4.30 and 6.93 min respectively. In the HPTLC method, the chromatograms were developed using a mobile phase of ethyl acetate: methanol: ammonia (8.5:1:0.5 v/v) on precoated plate of silica gel 60 F_{254} and quantified by densitometric absorbance mode at 285 nm. The R_f of HCT and NEB-H were 0.21 and 0.41 respectively. Recovery studies of 98.88-102.41%, percentage relative std deviation of not more than 0.8 and correlation coefficient (linearity range) of 0.9954-0.9999 shows that developed methods were accurate and precise. These methods can be employed for the routine analysis of tablets containing NEB-H and HCT.

Keywords: Nebivolol hydrochloride, Hydrochlorothiazide, UV spectrophotometry, RP-HPLC, HPTLC.

Introduction

Nebivololhydrochloride[1] (NEB-H) is a benzopyran antihypertensive drug (β $_1$ blocker) and chemically it is a *α, α*' - [iminobis (methylene)] *bis* [6-flouro-3,4,-dihydro-2*H*-1-benzopyran-2-methanol hydrochloride. Reports are available for estimation of NEB –H by HPLC and other methods[2-5]. Hydrochlorothiazide[6] (HCT) is a 6-chloro-3, 4-dihydro-2*H*-1, 2, 4-benzothiadiazine-7-sulfonamide-1, 1-dioxide, which is used as a diuretics. Hydrochlorothiazide is official in IP, BP, USP and EP. Several methods such as HPLC, HPTLC, spectrophotometry and nonaqueous potentiometric titration[7-26]. The combination of nebivolol hydrochloride (NEB-H) and hydrochlorothiazide is newly introduced in market and used in the treatment of hypertension. Moreover the literature survey revealed that, so far no method has been reported for estimation of NEB –H and HCT in combined dosage form. Therefore, it was thought worthwhile to develop simple, precise, accurate UV-Spectrophotometric, HPLC and HPTLC method for the simultaneous estimation NEB-H and HCT in tablets.

Experimental

UV spectral measurement recorded in Shimadzu (Japan) 1700 UV-Visible spectrophotometer with auto corrected wavelength accuracy of ±0.3 nm and 1 cm UV matched quartz cells were used. LC system used consists of pump (model SHIMADZU: LC-20 AT *vp* with universal loop injector (Rheodyne 7725i) of injection capacity 20 µL. Detector consists of Photodiode array detector SPD-20 Avp, SHIMADZU; the column used was C_{18} (5 µm, 25 cm x 4.6 mm *i.d.*) phenomenex, USA at ambient temperature and computer based data station were used. Pre coated silica gel $60F_{254}$ on aluminium sheets (200 µm thick) of E-Merck, Germany were used as stationary phase. Pre-washing of plate was done with methanol and then it was activated by keeping in an oven at 115 °C for 10 min. Camag HPTLC System (with TLC Scanner), WinCATS Softwar V 4.0 and Linomat 5 as application device) used for the analysis.

Chemicals and reagents

Micro Laboratories Ltd., India, generously gifted pure NEB-H, HCT and ATR. Commercial tablets (two different brands) containing NEB-H (5 mg) and HCT (12.5 mg) were used for this study. Water, acetonitrile, ethyl acetate, methanol, used were of HPLC grade (E. Merck, Mumbai, India). All the other chemicals used were of analytical grade (E. Merck, India).

UV method

Two stock solutions were prepared by dissolving 5 mg and 12.5 mg of NEB-H and HCT in a 100 mL of methanol, respectively. Seven mixed standard solutions were prepared from the stock solutions with different concentration ranging from 10-50 µg/mL and 1-5 µg/mL of NEB-H and HCT respectively. All the mixed standard solutions were scanned over the range of 200-400 nm. From the overlain spectra of both drugs, (Figure 1) wavelengths 275 nm (isoabsorptive point) and 282.5 nm (λ_{max} of NEB-H) were selected for the formation of absorbance ratio equation. For calibration curve, stock solutions of NEB-H and HCT were appropriately diluted to obtain concentration range of 10-50 µg/mL and 1-5 µg/mL respectively. The absorbance of NEB-H measured at 282.5 and 275 nm and calibration curves were plotted. Similarly the absorbance of HCT measured at 282.5 and 275 nm, calibration curves were plotted. The absorptivities (A1%, 1 cm) of each drug at both the wavelengths were also determined.

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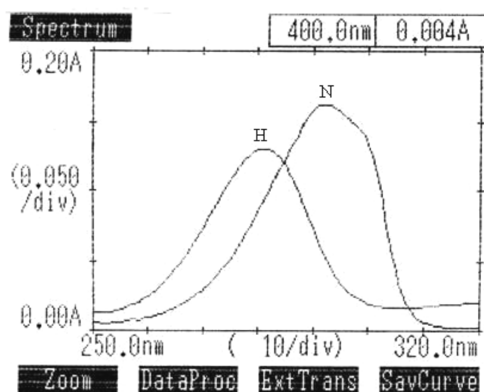

Figure 1. Overlain spectra of the tablet sample (NEB-H & HCT).

The absorbance and absorptivity values at the particular wavelengths were calculated and substituted in the following equation, to obtain the concentration.

$$C_{NEB\text{-}H} = (Q_M - Q_Y) \times A_1 / (Q_X - Q_Y) \times a_{x1}, \quad (1)$$

$$C_{HCT} = (Q_M - Q_X) \times A_1 / (Q_Y - Q_X) \times a_{x1} \quad (2)$$

Where, $C_{NEB\text{-}H}$, C_{HCT} are concentration of NEB-H, HCT respectively, A_1 is absorbance of sample at 275 nm, a_{x1} is the absorptivity of NEB-H at 275 nm, Q_x was obtained using the equation (absorptivity of NEB-H at 282.5 nm) / absorptivity of NEB-H at 275 nm, Q_y was obtained by using (absorptivity of HCT at 282.5 nm) / (absorptivity of HCT at 275 nm) and Q_M from (absorbance of sample at 282.5 nm) / (absorbance of sample at 275 nm).

For the selection of analytical wavelength in simultaneous equation method (Method 2) 271.5 and 282.5 nm (λ_{max} of HCT and λ_{max} of NEB-H) were selected. For calibration curves, stock solutions of NEB-H and HCT in the concentration of range of 10-50 µg/mL and 1-5 µg/mL respectively. The absorbance of NEB-H and HCT were measured at 282.5 and 271.5 nm, calibration curves were plotted. The absorptivities of both the drugs at both the wavelengths were determined.

The absorbance and the absorptivity values at the particular wavelength were calculated and substituted in the following equation, to obtain the concentration.

$$C_{NEB\text{-}H} = (A_1 a_{x2} - A_2 a_{x1}) / (a_{x2} a_{y1} - a_{x1} a_{y2}) \quad (3)$$

$$C_{HCT} = (A_2 a_{y1} - A_1 a_{y2}) / (a_{x2} a_{y1} - a_{x1} a_{y2}) \quad (4)$$

Where, $C_{NEB\text{-}H}$, C_{HCT} are concentration of NEB-H and HCT respectively, A_1 is the absorbance of sample at 282.5 nm, A_2 is the absorbance of sample at 271.5 nm, a_{x1} is the absorptivity of NEB-H at 282.5 nm and a_{x2} is the absorptivity of NEB-H at 271.5 nm, a_{y1} is the absorptivity of HCT at 282.5 nm and a_{y2} is the absorptivity of HCT at 271.5 nm. Twenty Tablets of two brands label claim 5 mg of NEB-H and 12.5 mg of HCT were weighed, average weight determined and finely powdered. Appropriate quantity of powder from each tablet equivalent to 12.5 mg of HCT was accurately weighed and following standard addition method (due to low absorbance), 120 mg of NEB-H was accurately weighed and added to achieve 10:1 ratio (NEB-H & HCT) shaken vigorously for 15 min and filtered. Necessary dilutions of filtrate were made with Methanol to get final concentration 10 µg/mL of NEB-H and 1 µg/mL of HCT. Absorbance of this solution was measured at 282.5, 275, and 271.5 nm a nd values were substituted in the respective formulae (Method 1 & 2) to

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obtain concentration and the results are shown in Table 1 and performing recovery studies by standard addition method in which pre-analysed samples were taken and standard drug was added at different levels carried out validation of proposed method. Results are shown in Table 3.

Table 1. Analysis of formulation by UV.

Samples	Label claim, mg / tab	*Assay mean % ± S.E.M		%RSD	
Tablet A		Method 1	Method 2	Method 1	Method 2
NEB-H	5.0	99.71 ± 0.274	100.2 ± 0.321	0.477	0.546
HCT	12.5	99.41 ± 0.103	98.97 ± 0.224	0.196	0.385
Tablet B					
NEB-H	5.0	100.23 ±0.262	100.75±0.157	0.453	0.312
HCT	12.5	100.25 ±0.385	99.75 ± 0.382	0.726	0.678

Method-1 Absorbance Ratio Method, Method-2 Simultaneous equation method.
**Each value is a mean of six observations*

The overlain spectra of both the drugs showed that the peaks are well resolved, thus satisfying the criteria for obtaining maximum precision, based on absorbance ratio. The criteria being the ratios (A_2/A_1) / (a_{x2}/a_{x1}) and $(a_{y2}$ / $a_{y1})$ / (A_2/A_1) should lie outside the range 0.1-2.0 for precise determination of (Y) and (X) respectively. Where A_1/A_2 represents the absorbance of mixture at λ_1 and λ_2, a_{x1} and a_{x2} denote absoptivities of (X) at λ_1 and λ_2 and a_{y1} and a_{y2} denote absoptivities of (Y) at λ_1 and λ_2 respectively. In this context, the above criterion was found to be satisfied for NEB-H (X) and HCT (Y). Where λ_1 (275 nm) and λ_2 (282.5 nm) for Q-absorbance method, λ_1 (282.5 nm) and λ_2 (271.5 nm) for simultaneous equation method.

Two wavelengths that could serve as isoabsorptive points were 271.5 and 307 nm as determined by evaluation of overlain spectra. By comparing absorptivity of both the drugs at these wavelengths 275 nm was found suitable for the analysis. Since both the drugs gave same absorptivity at this wavelength. Hence 275 and 282.5 nm was selected for Q-absorbance equation.

In simultaneous equation method two wavelengths *i.e.* λ_{max} of both the drugs were required, the spectra of HCT showed three distinct peaks one at around 226, 271.5 and 317 nm. The 271.5 nm was selected for analysis of HCT. The λ_{max} of NEB-H was 282.5 nm, which was used for estimation.

HPLC method

The phosphate buffer was prepared by dissolving 5.244 g of dipotassium hydrogen phosphate in distilled water and made up to the volume 1000 mL. The drugs were resolved using a mobile phase of 30 mM of dipotassium hydrogen phosphate buffer: acetonitrile: triethylamine (50:50:0.1% v/v) with pH adjusted to 5.5 using orthophosphoric acid filtered using membrane filter and degassed. The flow rate was 1 mL/min and the effluents were monitored at 282 nm (Figure 2).

A stock solution was prepared by dissolving 5, 12.5 and 5 mg of NEB-H, HCT and ATR (internal standard) in 100 mL of mobile phase to obtain various concentration of NEB-H (5-25 μg/mL), HCT (12.5-62.5 μg/mL) and ATR (5 μg/mL) respectively. A volume of 20 μL of each sample was injected into column. All measurements were repeated six times for each concentration and calibration curve was constructed by plotting peak area ratio of analyte to internal standard *vs.* the corresponding drug concentration.

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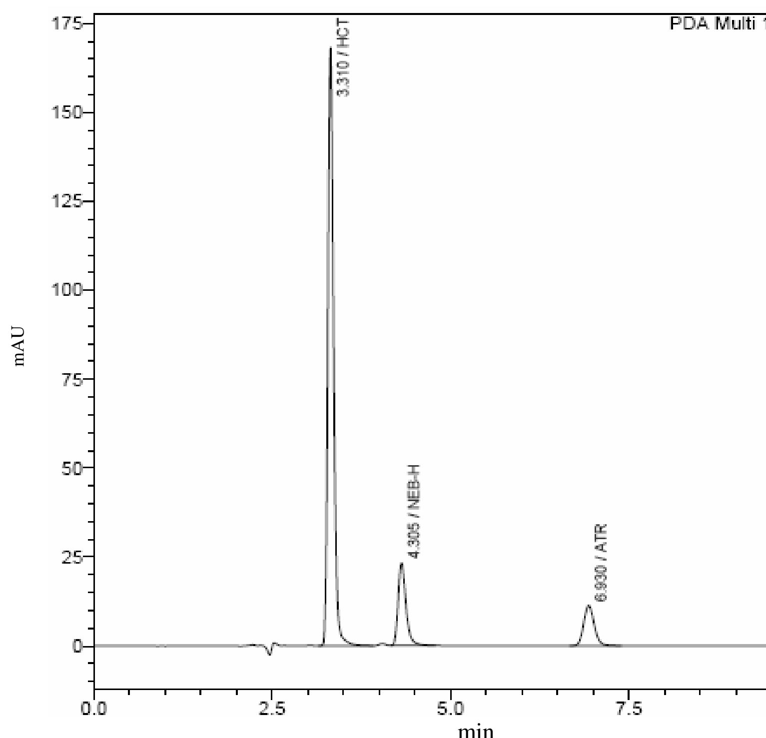

Figure 2. RP-HPLC Chromatogram of tablet sample with internal standard (NEB-H, HCT& ATR).

About 20 tablets were weighed and powdered. A powder equivalent to 12.5 mg of HCT was weighed accurately and transferred to a 100 mL volumetric flask. The tablet powder was dissolved in the mobile phase and filtered through a membrane filter (0.45 μm). The sample solution was suitably diluted and used for analysis. Twenty microlitres of standard and sample solutions were injected, respectively, under specified conditions and scans were recorded. Each solution was run thrice at an interval of 20 min to ensure the elution of earlier injection. The amount of NEB-H and HCT present per tablet was calculated by comparing the peak area sample with that of standard. The stability[27] sample in mobile phase was analysed after 24 h; it was found that there was no change in the analytical parameters, which was indicative of stability of all of these drugs in the solvents employed for the analysis. The quantification data and system suitability data are presented in Table 2 & 4.

HPTLC method

The drugs were resolved using a mobile phase of Ethyl acetate: Methanol: Ammonia (8.5:1:0.5 v/v),10 min time saturation with filter paper was selected because it gave compact spots and good resolution between analytes and good separation from solvent front and sample application positions. Development chamber (20x10 cm), migration distance (80mm), band length (8 mm), slit dimension (6x0.30 mm), temperature 26.4 ^{o}C, humidity 61% and UV detection was carried out at 285 nm Figure 3.

A stock solution was prepared by dissolving 10 mg and 25 mg of NEB-H and HCT in 100 mL of mobile phase. The stock solution were further diluted with methanol to obtain various concentration of 100-500 ng/mL and 250-1250 ng/L for NEB-H and HCT respectively. All the sample solution was applied on the TLC plate using LINOMET 5

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automatic device and detected. All measurements were repeated six times for each concentration and calibration curve was constructed by plotting peak area *vs.* the corresponding drug concentration.

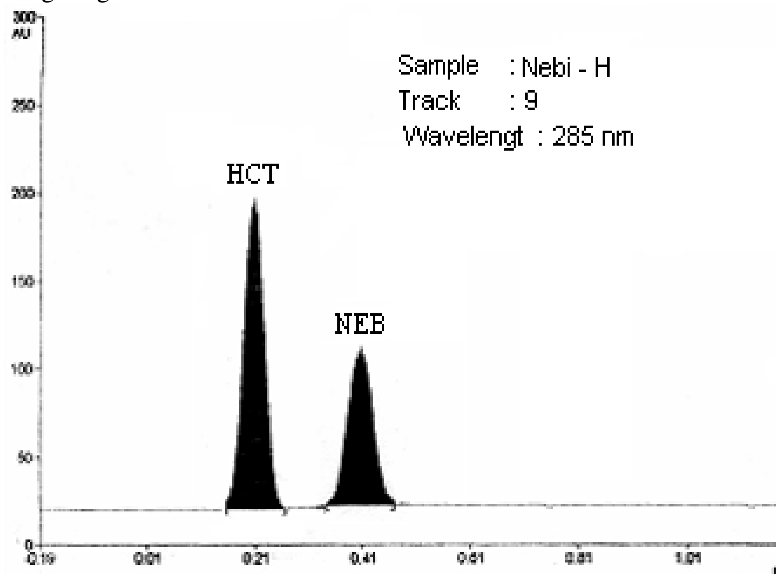

Figure 3. HPTLC Chromatogram of tablet sample (NEB-H & HCT)

The sample prepared as that of HPLC method and filtered through Whatman filter paper. The sample solution was suitably diluted and used for analysis. Two microlitres of standard and sample solutions were applied as band 8 mm at 8 mm interval under stream of nitrogen. The developed chromatograms were evaluated by scanning in densitometric mode at 285 nm. The amount of NEB-H and HCT present per tablet was calculated by comparing peak area pf sample with that of standard. The analytical data are represented in Table 2.

Table 2. Analysis of formulation by RP-HPLC & HPTLC.

Samples	Label claim, mg / tab	RP-HPLC	HPTLC	RP-HPLC	HPTLC
		*Assay mean % ± S.E.M	*Assay mean % ± S.E.M	%RSD	%RSD
Tablet A					
NEB-H	5.0	101.21 ± 0.416	102.41 ± 0.124	0.793	0.239
HCT	12.5	99.69 ± 0.269	98.88 ± 0.254	0.507	0.497
Tablet B					
NEB-H	5.0	99.67 ± 0.362	99.76 ± 0.421	0.719	0.812
HCT	12.5	100.55 ± 0.216	98.99 ± 0.216	0.425	0.409

** Each value is a mean of six observations.*

Recovery studies

Recovery studies were carried out by adding known quantities of standard at different levels to the pre-analysed sample to study the linearity, accuracy and precision of the proposed methods. The recovery studies also reveals whether there is a positive or negative influence on the quantification parameters by the additives usually present in dosage forms. The recovery study data are presented in Table 3.

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Table 3. Recovery studies of nebivolol hydrochloride and hydrochlorothiazide.

Samples	Label claim, mg	Fortified amount, mg	% Recovery*		
Tablet A			UV	RP-HPLC	HPTLC
NEB-H	5.0	1.5	98.88	101.13	98.40
		3.0	98.33	100.90	99.53
HCT	12.5	4.0	99.98	99.98	100.92
		8.0	100.25	100.30	98.82
Tablet B					
NEB-H	5.0	1.5	99.87	99.76	99.89
		3.0	98.89	100.64	100.86
HCT	12.5	4.0	99.86	99.33	99.76
		8.0	98.43	100.46	98.43

** Each value is a mean of six observations*

Results and Discussion

In UV spectrophotometric absorbance ratio method, the linearity of NEB-H and HCT was 10-50 µg/mL (r = 0.9999), 1-5 µg/mL (r = 0.9994), respectively. The recovery values were 98.72-100.15% with percentage relative standard deviation (%RSD) of <0.74.In simultaneous equation method NEB-H (r = 0.9992) and HCT (r = 0.9999) respectively. The recovery values were 99.41-100.75% with percentage relative standard deviation (%RSD) of not more than 0.8.

In the RP-HPLC method, system suitability (Table 4) was applied to a representative chromatograph to check various parameters such as efficiency, resolution and peak tailing which was found to be complying with BP requirements. The retention time of HCT, NEB-H and ATR (internal standard) was 3.31, 4.30 and 6.93 min respectively, with linearity range of 12.5-62.5 µg / mL (r = 0.9997) and 5-25 µg / mL (r = 0.9994) respectively. The recovery values were 99.67 - 100.75 with percentage relative standard deviation (%RSD) of not more than 0.8.

Table 4. System suitability and validation parameters for RP-HPLC.

Validation Parameters	HCT	NEB-H
Linearity range, µg/mL	12.5-62.5	5-25
r	0.9997	0.9994
LOD, ng /mL	10	5
LOQ, ng /mL	50	25
Intra day, % RSD*	0.6373	0.4575
Inter day, % RSD*	0.6453	0.6727
Repeatability, % RSD*	0.4820	0.3447
Accuracy	99-100 %	99 – 101%
Peak purity index	1.0000	1.0000
Resolution factor(R_s)	-	5.383
Asymmetry factor(A_s)	0.95	
No.of theoritical plates(N)	6952	6671
Capacity factor (K')	-	0.301
High equivalent to theoritical plates(HETP)	21.575	22.482
Tailing factor	1.327	1.423
Seletivity factor(α)	3.639	

** Each value is a mean of six observations.*

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In the HPTLC method, the R_f of HCT and NEB-H was 0.21 and 0.41 respectively with a linearity range of 250-1250 ng/mL (r = 0.9982) and 100-500 ng/mL (r = 0.9954) respectively. The recovery values were 98.88-102.41 with percentage relative standard deviation (%RSD) of not more than 0.9.

The proposed methods for the quantification of NEB-H and HCT in different brands of tablets were silmple, precise, accurate, rapid and selective. The methods are linear in the concentration range reported. The developed method are free from interference due to the excipients present in various brands of tablets and can be used for routine simultaneous quantitative estimation of NEB-H and HCT in tablets. In conclusion, the results have shown that HPLC method is best for a simultaneous quantification of NEB-H and HCT in tablets.

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