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Development and validation of RP-HPLC method for simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in combined tablet dosage form

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Abstract: The objective of the current study was to develop a simple, accurate, precise and rapid reversed phase high performance liquid chromatographic (RP-HPLC) method with subsequent validation using ICH suggested approach for the determination of antihypertensive pharmaceutical dosage form containing binary mixture of Nebivolol hydrochloride (NEB) and Hydrochlorothiazide (HCZ). The proposed RP-HPLC method utilizes a LiChrosorb^R C18, 5µm, 250mm × 4.6mm i.d. column in isocratic mode, mobile phase consisting of methanol and water in the proportion of 80:20 (v/v) with an apparent pH adjusted to 7.0 using 1.0 N potassium hydroxide solution. The column effluents were subjected to UV detection at 286 nm using a UV detector L-7400. The average retention times were 3.67 ± 0.0398 min and 1.64 ± 0.0052 min for NEB and HCZ respectively at a flow rate of 1.6 ml/min. The linearity of the proposed method was investigated in the range of 2-40 µg/ml (r = 0.9998) for NEB and 2-70 µg/ml (r = 0.9995) for HCZ respectively. The percentage mean recovery was found to be 99.47 for NEB and 100.04 for HCZ. Also the method was statistically validated for its linearity, accuracy and precision. Both inter-day and intra-day variation was found to be showing less % RSD value indicating high grade of precision of the method.

Keywords: RP-HPLC, Nebivolol hydrochloride, Hydrochlorothiazide, UV detection, Validation**.**

1. Introduction

Nebivolol hydrochloride (NEB) is chemically described as (±)-[2R*[R*[R*(S*)]]]- α, α' - [Iminobis (methylene)] bis [6-fluoro- 3, 4- dihydro- 2H-1-benzopyran- 2-methanol] hydrochloride [1,2]. It is a racemate of two enantiomers with four chiral centers, steriochemically designated as SRRR- Nebivolol (D-NEB) and RSSS-Nebivolol (L-NEB) respectively (Figure.1). The RSSS enantiomers possess a favourable heamodynamic profile. It is a cardio selective third generation β_1 receptor blocking agent. It blocks the β adreno-receptor effect of adrenaline and nor-adrenaline, reducing heart rate, force of myocardial infarction, decreases systemic blood pressure and increases diastolic pressure. In addition to adrenergic blocking property it possesses additional

vasodilating activity mediated by L-argenine nitric oxide pathway, increasing the bioavalability of nitric oxide (NO) which is the major endothelium derived vasoactive compound that produces vasodilation by enhancing cyclic guanosine monophosphate and also inhibits platelets aggregation and smooth muscle cell proliferation. After oral administration of NEB, the blood: drug concentration reaches peak value within 0.5-2 hrs. The oral bioavailability of the drug averages 12 % in fast metabolizers and 96 % in slow metabolizers. Also it has plasma protein binding property [3,4].

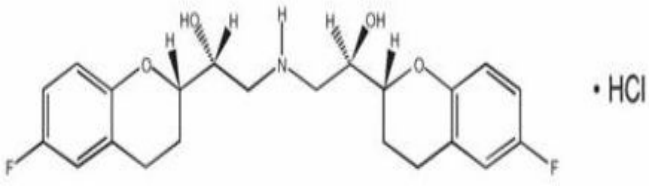

Figure 1: Structural formulae for Nebivolol hydrochloride (MW= 441.9).

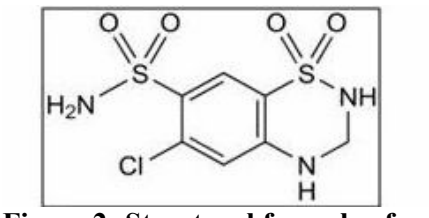

Figure 2: Structural formulae for Hydrochlorothiazide (MW= 297.73).

Hydrochlorothiazide (HCZ) is chemically described as 6-Chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide [1,2] (Figure.2). It belongs to the class of thiazide diuretics, widely used in the treatment of hypertension and oedema associated with mild to moderate congestive heart failure. It increases the rate of urine excretion by the kidneys through decreased tubular reabsorption of sodium and chloride ions and by increasing osmotic transport of water to renal tubules, which in turn lowers the cardiac output and blood pressure. On prolonged thiazide treatment plasma volume and ECF returns to normal but their hypotensive effect continues due to reduced sensitivity of vascular beds to circulating catecholamine and angiotensin [3,4]. It is orally well absorbed, showing its peak effect within 4-6 hrs and passing off by 10-12 hrs. It is relatively soluble in water and most rapidly excreted in urine in unchanged form [5-7].

HCZ in combination with NEB potentiates the antihypertensive activity showing synergestic effect in reducing systolic and diastolic blood pressure. In addition to excess reduction in blood pressure the combination of NEB and HCZ is safe, well tolerated with lower incidence of adverse effects and a neutral impact on lipid and glucose metabolism.

Literature survey reveals that few methods have been reported for the determination of HCZ or NEB individually in biological fluids or in combination with other drugs in pharmaceutical dosage forms [8-23]. But no method has been developed for simultaneous estimation of NEB and HCZ in combined dosage form. The present manuscript describes a sensitive, simple, precise and accurate isocratic RP-HPLC method for simultaneous estimation of NEB and HCZ in combined dosage form with subsequent validation as per ICH guidelines [24-27].

2. Experimental

2.1. Chemicals and reagents

The working standards of NEB and HCZ were generous gift obtained from Alembic Pharmaceuticals Ltd. (Baroda, India). The combination formulation of NEB and HCZ (Label claim: Nebivolol 5 mg, as Nebivolol hydrochloride and Hydrochlorothiazide 12.5 mg), Nebicard-H tablets (Torrent Pharmaceuticals Ltd.) were purchased from the local market. Methanol (HPLC grade) and double distilled water were obtained from E. Merck Ltd. Potassium hydroxide was obtained from S. D. Fine Chemicals Ltd.

2.2. HPLC instrumentation

The liquid chromatographic system consisted of following components: Quaternary gradient HPLC system – Merck Hitachi, double reciprocating pump L-7100, variable wavelength programmable UV detector L-7400, a universal injector 77251 (Rheodyne) with injection volume 20 µl. Chromatographic analysis was performed using Merck-Hitachi HSM software on a LiChrosorb^R C18 column, with 250mm × 4.6mm i.d. and 5µm particle size.

2.3. Preparation of mobile phase and standard stock solution

Both, HPLC grade methanol and Double distilled water were ultrasonicated for 20 minutes on ultrasonicator and filtered through 0.45µm Nylon 66 (N_{66}) 47 mm membrane filter paper seperately. The mobile phase was prepared by mixing previously ultrasonicated and filtered solvents, methanol and water in the ratio of 80:20 (v/v) with an apparent pH adjusted to 7.0 using 1.0 N potassium hydroxide solution.

The standard stock solutions 100 µg/ml each of NEB and HCZ were prepared separately by dissolving the working standards in small proportions of mobile

phase and later diluted to desired volume with the same. The standard calibration solutions of NEB and HCZ having concentration range 2-40 and 2-70 µg/ml respectively were prepared by diluting appropriate aliquots of the standard stock solutions with the mobile phase.

2.4. Chromatographic conditions

The mobile phase consisting of methanol and water in the ratio of 80:20 (v/v) with an apparent pH adjusted to 7.0 using 1.0 N potassium hydroxide solution was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.45 µm Nylon 66 (N66) 47 mm membrane filter paper. The measurements were carried out with an injection volume of 20µl, flow rate was set to 1.6 ml/min and UV detection was carried out at 286 nm.. All determinations were performed at ambient column temperature (27°C). The chromatograms of the prepared standard stock solutions of NEB and HCZ were individually recorded under the above optimized chromatographic conditions (**Figure. 3 and Figure. 4).**

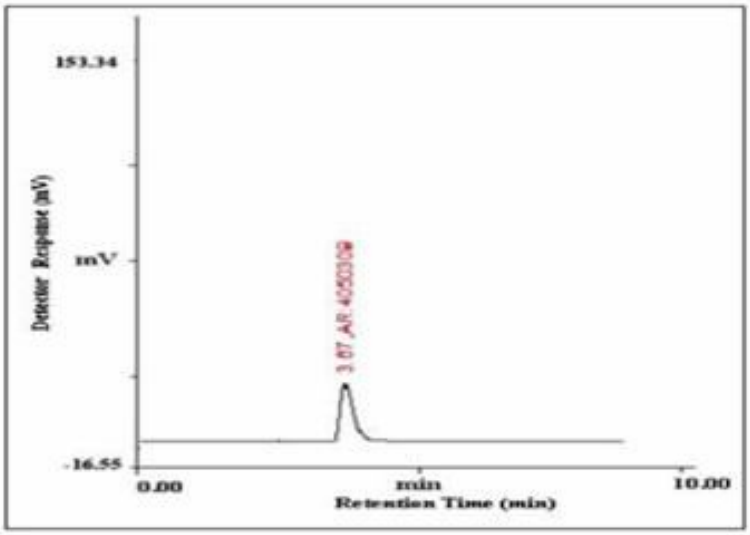

Figure 3: RP-HPLC chromatogram of a 20 µl injection containing 20 µg/ml of Nebivolol hydrochloride.

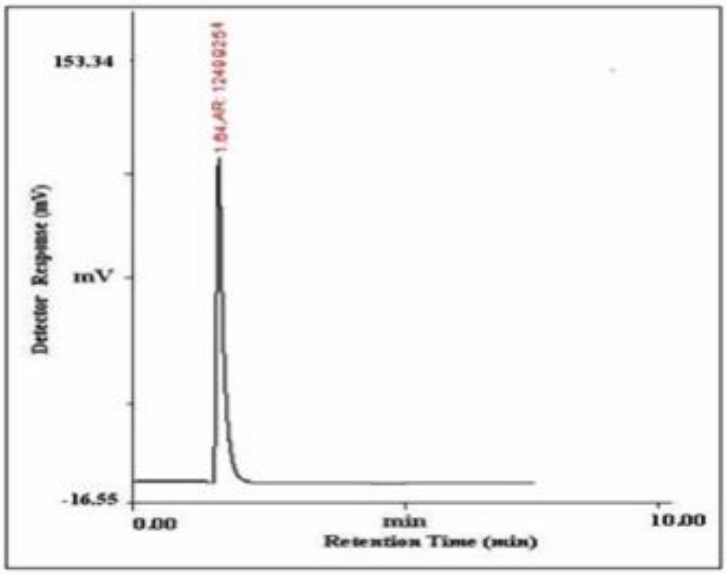

Figure 4: RP-HPLC chromatogram of a 20 µl injection containing 50 µg/ml of Hydrochlorothiazide.

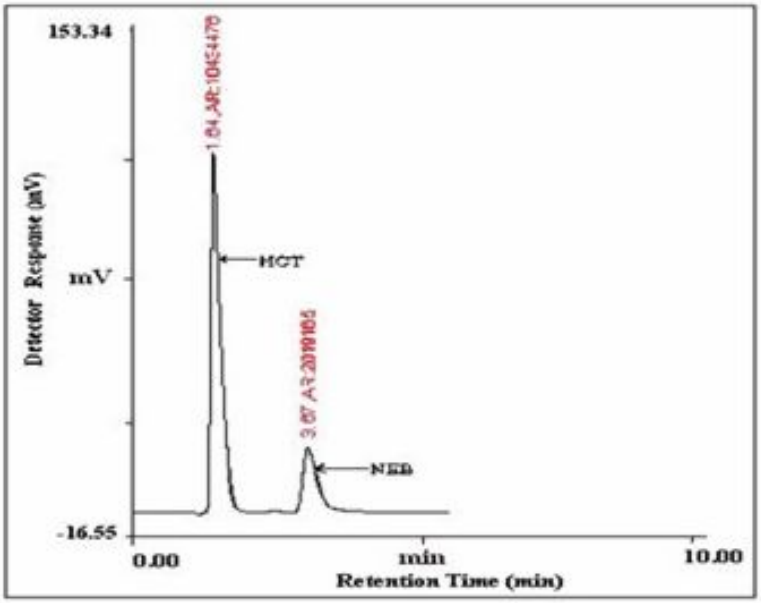

Figure 5: RP-HPLC chromatogram of a 20 µl injection containing mixture of 20 µg/ml of Nebivolol hydrochloride and 50 µg/ml of Hydrochlorothiazide in tablet formulation.

2.5. Analysis of tablet dosage form

Twenty tablets were weighed, their mean weight was determined and finally they were crushed in mortar to obtain a fine powder. An amount of powdered mass equivalent to one tablet content was transferred into a 100 ml volumetric flask and dissolved in sufficient quantity of mobile phase. The contents were ultrasonicated for 20 minutes and the final volume was made up to the mark with mobile phase. The prepared solution was then filtered through 0.2 µm Nylon 66 (N66) 47 mm membrane filter paper and was used as standard stock solution. Appropriate aliquot was pippetted out from the standard stock solution and was further diluted with the mobile phase to obtain a mixture containing 20 µg/ml of NEB and 50 µg/ml of HCZ. A 20 µl volume of the sample mixture was injected in to the sample injector and their chromatograms were recorded under the optimized chromatographic conditions (**Figure. 5).** The area of each peak was determined at 286 nm and the amount of drug present in the sample mixture was determined.

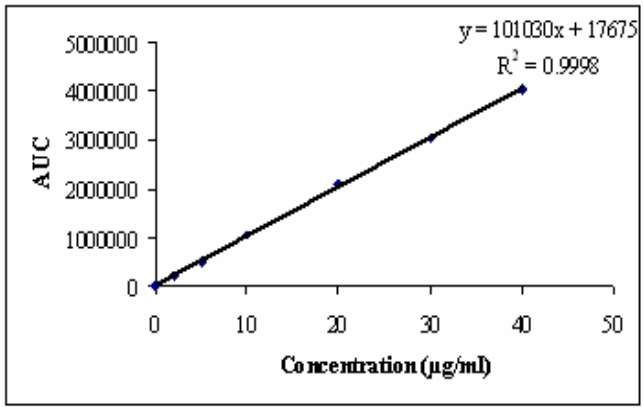

Figure 6: Calibration curve of Nebivolol Hydrochloride at 286 nm by RP−HPLC Method.

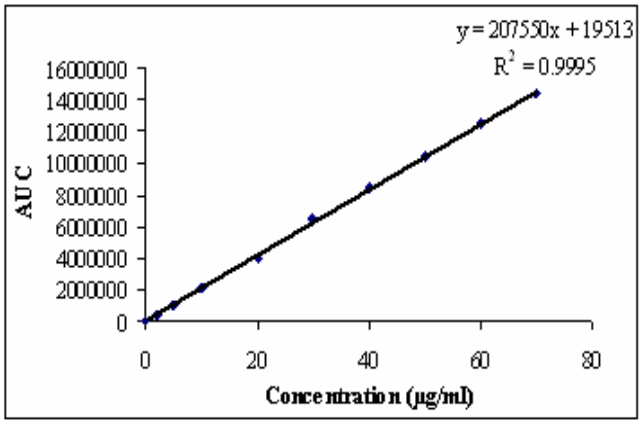

Figure 7: Calibration curve of Hydrochlorothiazide at 286 nm by RP−HPLC Method.

3. Result and discussion

3.1. Method development and optimization

A LiChrosorb^R C18, 5 µm, 250mm×4.6mm i.d. column (E. Merck Ltd. India) in isocratic mode maintained at ambient temperature (27°C) was used for the separation and method validation was performed for the determination of NEB and HCZ in Nebicard-H tablets. LiChrosorb^R column is a ultra pure silica gel based column possessing excellent chemical and mechanical stability and provides good peak symmetry for the analytes. The composition, pH and the flow rate of the mobile phase were optimized to obtain good resolution. A mobile phase consisting of methanol and water in the ratio of 80:20 (v/v), with pH adjusted to neutral using 1.0 N potassium hydroxide solution, set at a flow rate of 1.6 ml/min was selected for the chromatographic analysis. Under above described experimental conditions, all the peaks were well defined and free from tailing. The effect of small deliberate changes in the mobile phase composition, flow rate and column temperature on results was evaluated as a part of testing for methods robustness. The peak homogeneity was expressed in terms of peak purity values and was obtained directly from the spectral analysis of the sample.

3.2. Method validation

The developed analytical method was subjected to validation with respect to various parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery studies, specificity and reproducibility and robustness / ruggedness as per the ICH guidelines [24-27].

3.2.1. Linearity

Linearity was established by least square regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 2-40 µg/ml and 2-70 µg/ml for NEB and HCZ respectively. Peak areas of NEB and HCZ were a plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves. Correlation coefficients (n = 6) were found to be more than 0.999 for both the drugs with (%) RSD value 0.0167% and 0.0055% for NEB and HCZ respectively [24,25]. Typically, the regression equations were found to be: y = 101030x + 17675 (r = 0.9998) for NEB and y = 207550x + 19513 (r = 0.9995) for HCZ respectively. The Area under curve versus concentration regression data, including the data of calibration equations and correlation coefficients obtained for both drugs are listed in **Table 1.**

3.2.2. LOQ and LOD

The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. LOD was determined on the basis of signal to noise ratios and was determined using analytical response of three times the background noise. Both LOQ and LOD were calculated on the peak area using the following equations:

$LOQ = 10 \times N/B$ $LOD = 3 \times N/B$

Where, N is the standard deviation (SD) of the peak areas (triplicate injections) of the drug, B is the slope of the corresponding calibration curve. The limit of quantification and the limit of detection of NEB and HCZ were found to be 0.0309 µg/ml and 0.0046 µg/ml, 0.0102 µg/ml and 0.0015 µg/ml, respectively [26,27].

Table 1: Statistical analysis of the calibration curves of NEB and HCZ respectively

Parameters	NEB	HCZ
Range (µg/ml)	2.0 – 40.0	2.0 – 70.0
Slope*	101030	207550
SD on slope*	1069.4	636.13
Intercept*	17676	19513
SD on intercept*	312.13	96.29
Correlation coefficient (r)*	0.9998	0.9995
LOQ (µg/ml)**	0.0309	0.0046
LOD (µg/ml)**	0.0102	0.0015

Where, *n = 6 and **n = 3

3.2.3. Accuracy

Accuracy of the method was determined by interpolation of peak areas of six replicate samples (n = 6) of tablets formulation containing 20 µg/ml of NEB and 50 µg/ml of HCZ, from the calibration curve that had been previously prepared. The accuracy data for the assay following the determination of each component of interest is summarized in **Table 2.**

3.2.4. Precision

Intra-day precision was estimated by assaying the quality control sample of the tablet formulation containing 20 µg/ml of NEB and 50 µg/ml of HCZ, six times (results averaged for statistical evaluation) in the same analytical run. The statistical validation data for intra day precision is summarized in **Table 3.**

Inter-day precision was evaluated by analyzing a set of quality control samples of the tablet formulation containing 20 µg/ml of NEB and 50 µg/ml of HCZ, six levels analyzed on three consecutive days in the same analytical runs. The samples were prepared in advance and stored at 5°C. The statistical validation data (results averaged for statistical evaluation) for intra day precision is summarized in **Table 4.**

Table 2: Statistical validation data for accuracy determination (n = 6)

	Interpolated concentration (mean ± SD) *	RSD (%)*	SE (%)*
Nebivolol hydrochloride (µg/ml)			
20	19.978 ± 0.1626	0.8138	0.0664
Hydrochlorothiazide (µg/ml)			
50	49.946 ± 0.2530	0.5065	0.1033

Where, *n = 6

Table 3: Statistical validation data for determination of intra-day precision (n = 6)

	Interpolated concentration (mean ± SD) *	RSD (%)*	SE (%)*
Nebivolol hydrochloride (µg/ml)			
20	19.95 ± 0.0459	0.2301	0.0187
Hydrochlorothiazide (µg/ml)			
50	50.02 ± 0.0524	0.1047	0.0214

Where, *n = 6

Table 4: Statistical validation data for determination of inter-day precision (n = 3)

	Interpolated concentration (mean ± SD) *	RSD (%)*	SE (%)*
Nebivolol hydrochloride (µg/ml)			
20	19.91 ± 0.1611	0.8091	0.093
Hydrochlorothiazide (µg/ml)			
50	50.04 ± 0.0809	0.1617	0.0467

Where, *n = 3

3.2.5. Recovery

Recovery studies were also performed to determine the accuracy and precision of the proposed RP-HPLC method. Recovery experiments were performed at three levels, in which the sample stock solutions were spiked with standard drug solution containing 80%, 100% and 120% of the labeled amount of both the drugs (5 mg NEB and 12.5 mg HCZ) in tablet formulation. Three replicate samples of each concentration levels were prepared and the percentage recovery at each level (n = 3) [24,25], and mean % recovery (n = 9) were determined and summarized in **Table 5.** The mean (%) recovery was found to be 99.58% and 100.04% for NEB and HCZ respectively.

3.2.6. Specificity and reproducibility

The specificity of the RP-HPLC method was determined by complete separation of NEB and HCZ, with respect to various system suitability parameters like retention time (t_R), resolution (R_S) and tailing factor (T_f) as summarized in **Table 6** [26,27]. Here tailing factor for peaks of NEB and HCZ was less than 2% and resolution was more than 1%. The average retention times for NEB and HCZ were found to be (3.67 min ± 0.0398 and 1.64 min ± 0.0052) respectively for six determinations. The peaks obtained for NEB and HCZ were sharp and have a clear baseline separation indicating high degree of specificity.

The reproducibility of proposed RP-HPLC method was evaluated by analyzing the samples by two different analysts on different days using two columns containing the same brand of packing materials under the same chromatographic conditions. The two sets of data obtained were subjected to t-test analysis at 95% confidence level. The result of t-test analysis was found to be 0.9972, indicating no statistically significant difference.

Table 5: Recovery of NEB and HCZ in spiked standard drug solution

Level of (%) Recovery	Amount present (mg)		Amount added (mg)		Amount found (mg)		Recovery (%)	
	NEB	HCZ	NEB	HCZ	NEB	HCZ	NEB	HCZ
Level 1	5.0	12.5	4.0	10.0	8.89	22.50	98.78	100.02
(80%)	5.0	12.5	4.0	10.0	9.05	22.58	100.61	100.37
	5.0	12.5	4.0	10.0	8.99	22.57	99.96	100.31
Level 2	5.0	12.5	5.0	12.5	9.89	25.01	98.91	100.05
(100%)	5.0	12.5	5.0	12.5	9.91	25.03	99.10	100.10
	5.0	12.5	5.0	12.5	10.05	25.09	100.53	100.35
Level 3	5.0	12.5	6.0	15.0	10.86	27.27	98.75	99.16
(120%)	5.0	12.5	6.0	15.0	10.88	27.46	98.90	99.86
	5.0	12.5	6.0	15.0	10.96	27.55	99.68	100.19
	Mean (%) recovery*					RSD (%)*		
NEB	99.58					0.7745		
HCZ	100.04					0.2915		

Where, *n = 9

Table 6: Summary of system suitability parameters of NEB and HCZ

Parameters	NEB	HCZ
Retention time (min)	3.67 ± 0.0398	1.64 ± 0.0052
Resolution factor	3.123	3.123
Tailing factor	1.5	1.0
Capacity factor	3.77	1.79
Separation factor	2.11	2.11

Table 7: Robustness evaluation of the proposed RP-HPLC method

Parameters	Modification	Retention time (min)		(%) Recovery	
		NEB	HCZ	NEB	HCZ
Flow rate (ml/min)	1.5	3.72	1.66	101.54	98.40
	1.6	3.67	1.64	100.04	100.02
	1.7	3.60	1.61	99.55	98.49
(%) of methanol	79	3.64	1.65	101.01	99.75
in mobile phase	80	3.67	1.64	100.01	100.02
	81	3.63	1.60	98.84	98.49
Column Temperature	25	3.65	1.65	99.75	00.56
(^{o}C)	27	3.67	1.64	100.04	100.02
	29	3.62	1.63	101.21	98.35

3.2.7. Robustness

The evaluation of robustness of the proposed RP-HPLC method was considered during the development phase. The robustness of the method was investigated by subjecting the sample analysis to small deliberate variations in the analytical conditions including flow rate, composition of mobile phase and column temperature. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators had proven that the method was robust and the data are summarized in **Table 7.**

4. Conclusion

The validation results obtained confirm the suitability of the proposed RP-HPLC method for simple, accurate and precise analysis of NEB and HCZ in pharmaceutical preparations. The proposed method does not need prior separation of NEB and HCZ before analysis. In addition the proposed method is suitable for application without interference of excepients and can be applied directly to the commercial preparation without previous treatment.

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References

[1] M.J. O Neil, A. Smith, P.E. Heckelman, S. Budavari, The Merck Index, an Encyclopedia of Chemicals, Drugs and Biologicals, 13th ed., Merck & Co. Inc., White House Station, New Jersey, 2001, pp. 854, 1152.

[2] S.C. Martindale the Complete Drug Refrence, 34th ed., Pharmaceutical Press,London,2005,pp. 907.3, 938.2.

[3] C. Dollery, Therapeutic Drugs, second ed., Churchill Livingstone, UK, 1999, pp. H-52, H-57.

[4] A.G. Goodman, L.S. Gilman, in: A.G. Gilman, T.W. Rall, A.S. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics 8th ed., Pergamon Press, Oxford, 1990, pp. 286, 847.

[5] United States Pharmacopoeia, Twenty Second Revision, Unites states Pharmacopoeial Convention, Rockville, MD, 2003.

[6] British Pharmacopoeia, Her Majesty's Stationery Office, London, Vol-I, 1993.

[7] Indian Pharmacopoeia, The Controller of Publication, New Delhi, Vol-I, 1996.

[8] N. Erk, J. Pharmaceut. Biomed. Anal. 24 (2001) 603.

[9] N. Erk, Anal. Lett. 35 (2002) 283.

[10] K.R. Rajeswari, G.G. Sankar, A.L. Rao, D.B. Raju, J.S. Rao, Asian. J. Chem. 17 (2005) 1259-1263.

[11] J. Hendrickx, H. Bockx, C. Zwijsen, C. Borgmans, G. Mannens, W. Meuldermans, J. Heykants, J Chromatogr A. 729 (1996) 341-354.

[12] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, M. Koteshwara, S. Manoj, M. Santosh, J. Chidambara, S.Wishu,J.Chromatogr.B.809 (2004) 243-249.

[13] N.V.S. Ramakrishna, K.N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D.P. Varma, J. Pharm. Biomed. Anal. 39 (2005) 1006-1013.

[14] P.S. Selvan, K.V. Gowda, U. Mandal, W.D.S. Solomon, T.K. Pal, J. Chromatogr. B. 72 (2007) 127.

[15] O.A. Razak, J. Pharm. Biomed. Anal. 34 (2004) 433-440.

[16] A.S. Christophersen, K.E. Rasmussen, B. Salvesen, J. Chromatogr. A.132 (1997) 91-97.

[17] S. Hillaert, K.D. Grauwe, W.V. Bossche, J. Chromatogr. A. 924 (2001) 439-449.

[18] D. Zendelovska, T. Stafilov, P. Milosevski, J. Biomed. Chromatogr. 18 (2004) 71-76.

[19] K. Richter, R. Oertel, W. Kirch, J. Chromatogr. A. 1996; 729: 293-296.

[20] K.B. Alton, D. Desrivieres, J.E. Patrick, J. Chromatogr. B. 374 (1986) 103-110.

[21] S.S. Zarapaker, S.H. Rane, Indian Drugs. 37 (2000) 589-593.

[22] R. Sahu, B. Vandana, Ind. J. Pharm. Sci. 68 (2006) 764-767.

[23] S.B. Wankhede, M.R. Tajne, K.R. Gupta, S.G. Wadodkar, Ind. J. Pharm. Sci. 69 (2007) 298-300.

[24] ICH, Harmonised Tripartite Guideline, Text on Validation of Analytical Procedures, International Conference on Harmonization, Geneva, October 1994, pp. 21-39.

[25] S. Bolton, In., Pharmaceutical Statistics, third ed., Marcel Dekker Inc., New York, 2002, pp. 277-284.

[26] P.D. Sethi, Inc., High Performance Liquid Chromatography: Quantitative Analysis of Pharmaceutical Formulations, CBS Publishers and Distributors, New Delhi, 2001, pp. 215-263.

[27] L. George, N. Schmuff, HPLC Methods for Pharmaceutical Analysis, John Wiley and Sons, Inc. New York, 1996, pp. 682-698.

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