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Liquid chromatographic impurity profiling of Nebivolol Hydrochloride from bulk drug

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Abstract

Nebivolol hydrochloride was subjected to different ICH prescribed stress conditions of thermal stress, hydrolysis, oxidation and UV degradation. Two major degradants were detected by HPLC. For establishment of analytical assay, the reaction solutions in which different degradants were formed were separately used, and the separation was optimized by varying the HPLC conditions. Isocratic RP-HPLC separation was achieved on a C_{18} column (250 mm length ×4.6 mm internal diameter and 5 µm particle size) for both major degradants (impurities) of nebivolol hydrochloride by acid hydrolysis and by oxidation. The mobile phase comprising of methanol:water [80:20v/v, (pH 7.2, adjusted by adding 0.2 M glacial acetic acid into 0.2 M triethyl amine)] was used in both assays. The flow rate was adjusted to 1.0 ml/min and detection was performed at 222 nm using a UV detector. The pure impurities were synthesized by appropriate synthetic route at laboratory scale. Prior to spectroscopic characterization of impurities, they were separated and purified using pH partitioning and/or extraction recrystallization and/or chromatographic techniques. The pure impurities were characterized by spectral studies. The impurities appeared at relative retention time (RRT) of 0.69 min and 0.64 min for acid hydrolysis and oxidation of nebivolol hydrochloride respectively. A simple, precise, and accurate isocratic reversed phase stability indicating high performance liquid chromatographic assay method was developed and validated for determination of two identified impurities. The validation studies established a linear response of acid and oxidative degradation products (impurities). In the presence of nebivolol hydrocloride the limit of detection for its acid degradation product was 1.45µg/ml and that for its oxidative degradation product was 2.74µg/ml. The degradants produced as a result of stress studies and drug did not interfere with detection of each other, and the assay can thus be considered stability-indicating.

Keywords: β-blocker, degradation, impurity profiling, degradant, stability-indicating assay, liquid chromatography.

Introduction

The revised parent drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) requires that stress testing on the drug substance should be performed to establish stability characteristics and to support the suitability of the proposed analytical method. It is suggested that stress testing should include the effect of temperature, light, and oxidizing agents. It is also recommended that sample stability should be determined through the use of a validated stability testing method. Nebivolol hydrochloride has the chemical name 1-(6-fluorochroman-2-yl)-2-[(2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino] ethanol (NEB). Its molecular formula is $C_{22}H_{25}F_2NO_4$ and molecular weight is 405.45 g/mole. It is the most selective β_1-receptor antagonist currently available for clinical use [1]. NEB is a racemate of two enantiomers, D-NEB and L-NEB. D-NEB (the SRRR enantiomers represent the configuration at a particular chiral centre in the NEB) is a potent and cardio-selective β_1-adrenergic blocker, and L-NEB has a favourable and homodynamic profile [2-3]. NEB is a vasodilating β-blocker, which can be distinguished from other β-blockers by its hemodynamic profile [4]. It combines β-adrenergic blocking activity with a vasodilating effect mediated by the endothelial L-arginine nitric oxide pathway [5]. A high-performance thin-layer chromatographic (HPTLC) method was published for quantitation of NEB in its formulation [6], but the HPLC method has many advantages over the HPTLC method for quantitation. Moreover, HPLC is often the first choice of chromatographers compared to HPTLC. An HPLC method was reported in the literature for the determination of NEB in bulk and pharmaceutical dosage form [7]. The rapid quantitation of NEB in human plasma by HPLC/mass spectrometry was also reported [8]. This paper deals with the forced degradation of NEB under conditions such as acid hydrolysis, base hydrolysis, oxidation, thermal and UV stress. The aim of the current study was to develop a validated stability-indicating high-performance liquid chromatographic (HPLC) assay method for determination of acid degradant (NEBAD) and oxidant degradant (NEBO) as two identified impurities. Method validation was done according to ICH guidelines [9].

Results and Discussion

Degradation behaviour

In total, separately two major degradants were detected by HPLC on decomposition of the drug under acidic and oxidation conditions. The retention times (RT) and relative retention times (RRT) of the drug and the degradation products are listed in Table 1A and Table 1B. The degradation behaviour of the drug in individual stress conditions is outlined below:

Thermal stress

The exposure of the solid drug to 50^0C for 8 hrs did not result in significant decomposition. It indicated that NEB was stable to dry heat.

Hydrolysis

The drug degraded on heating at 70^0C for 1 hr in 0.1N HCl, forming major peak at RRT 0.69. The reaction in 0.1N NaOH at 70^0C for 1 hr did not result in significant degradation.

Oxidation
The drug was stable to 3% hydrogen peroxide at room temperature and no significant degradation was observed. However, decomposition occurred in 30% hydrogen peroxide, resulting in products resolving again at RRT 0.64.

UV degradation
The HPLC profile of light exposed drug sample was similar to those in the dark, indicating that light had no particular influence on the drug.

Development and optimization of the stability-indicating method
The acceptable separations with reasonable peak shapes were achieved by using mobile phase comprising of methanol:water [80:20v/v, (pH 7.2, adjusted by adding 0.2 M glacial acetic acid into 0.2 M triethyl amine)] and flow rate of 1.0 ml/min. The injection volume and detection wavelength were 20 µl and 222 nm, respectively.

Table 1A: Retention time and relative retention times of various peaks

Peak	Retention time (RT)	Relative retention time (RRT)
NEB	4.47	1.00
NEBAD	3.09	0.69

NEB- Nebivolol hydrochloride remained after acid hydrolysis; NEBAD- Nebivolol hydrochloride acid degradant.

Table 1B: Retention time and relative retention times of various peaks

Peak	Retention time (RT)	Relative retention time (RRT)
NEB	4.51	1.00
NEBO	2.91	0.64

NEB- Nebivolol hydrochloride remained after acid hydrolysis; NEBO- Nebivolol hydrochloride oxidation degradant

Validation of the developed stability-indicating method
The data obtained from linearity studies are given in Table 2. The response of the NEBAD and NEBO was strictly linear in the concentration range between 20 and 100µg/ml. The mean values (± R.S.D.) of slope, intercept and correlation coefficient were shown in Table 2. In the presence of NEB the limit of detection for its acid degradation product was found to be 1.45µg/ml and that for its oxidative degradation product was 2.74µg/ml. The limit of quantitation was found to be 4.41µg/ml and 8.31µg/ml for acid degradation product and oxidation degradation product of NEB respectively. The mean % R.S.D. values for intra and inter-day precision were shown in Table 3, confirming that the method was sufficiently precise. Good separation was achieved even when the procedure was repeated by a different person, thus confirming the reproducibility of the method.

Table 2: Linearity data on three different days by using mobile phase methanol:water [80:20v/v, (pH 7.2)]

Impurity	Regression parameter	Day 1	Day 2	Day 3	Mean±S.D.(% R.S.D.)
NEBAD	Slope	0.1917	0.1909	0.1898	0.1908±0.0009(0.4716)
	Intercept	-0.5217	-0.5017	-0.4809	-0.5014±0.0204(4.0686)
	R^2	0.9992	0.9994	0.9990	0.9992±0.0002(0.0200)
NEBO	Slope	0.0168	0.0168	0.0178	0.0171± 0.0005(2.9239)
	Intercept	-0.0305	-0.0328	-0.0360	-0.0331±0.0027(8.1570)
	R^2	0.9994	0.9992	0.9990	0.9992±0.0002(0.0200)

NEBAD- Nebivolol hydrochloride acid degradant.; NEBO- Nebivolol hydrochloride oxidation degradant.

Table 3: Reproducibility and precision data obtained during intra-day (n=6) inter-day (n=3) studies

Impurity	Actual Concentration(µg/ml)	Intra-day measured concentration (µg/ml)±S.D.(% R.S.D.)	Inter-day measured concentration (µg/ml)±S.D.(% R.S.D.)
NEBAD	30	30.26±0.2618(0.8651)	29.76±0.1627(0.5467)
	60	59.86±0.0358(0.0598)	60.26±0.2645(0.4389)
	90	89.58±0.3518(0.3927)	89.52±0.5515(0.6160)
NEBO	30	29.62±0.5507(1.8592)	29.66±0.3646(1.2292)
	60	60.46±0.2416(0.3996)	61.26±0.0657(0.1072)
	90	89.24±0.3686(0.4130)	89.75±0.2615(0.2913)

NEBAD- Nebivolol hydrochloride acid degradant.; NEBO- Nebivolol hydrochloride oxidation degradant.

Table 4: Recovery studies of NEBAD using nine different dilutions from degradation solution

Volume of acid degradation solution diluted to 10 ml	Actual added NEBAD concentration(µg/ml)	Measured of NEBAD concentration(µg/ml)	Recovery (%)
0.2	20	20.05	100.25
0.3	20	20.10	100.50
0.4	20	19.86	99.30
0.5	20	20.09	100.45
0.6	20	19.68	98.40
0.7	20	19.98	99.90
0.8	20	20.05	100.25
0.9	20	20.02	100.01
1.0	20	19.69	98.45
Average recovery			99.72

NEBAD- Nebivolol hydrochloride acid degradant.

As shown from the data in Table 4 and Table 5, good recoveries were made at the added concentration of 20µg/ml of NEBAD and NEBO for degradation solution of acid hydrolysis and degradation solution of oxidation, respectively. Figure 1 and Figure 2 shows that the method was selective to the drug as well as the degradation products. The system suitability parameters are given in Table 6 and Table 7.

Table 5: Recovery studies of NEBO using nine different dilutions from degradation solution

Volume of oxidation degradation solution diluted to 10 ml	Actual added NEBO concentration(µg/ml)	Measured of NEBO concentration(µg/ml)	Recovery (%)
0.2	20	19.76	98.80
0.3	20	20.35	101.75
0.4	20	19.88	99.40
0.5	20	19.88	99.40
0.6	20	19.86	99.30
0.7	20	20.08	100.40
0.8	20	19.64	98.20
0.9	20	20.35	101.75
1.0	20	19.68	98.40
Average recovery			99.71

NEBO- Nebivolol hydrochloride oxidation degradant.

Table 6: System suitability parameters for acidic degradation of NEB

Name of peak	R.T.	Theoretical plates	Selectivity	Capacity	Resolution	Asymmetry
NEBAD	3.09	3866.78	0.00	308.50	0.00	1.45
NEB	4.47	2361.22	1.45	447.33	4.90	1.54
DUT	8.47	6687.44	1.89	846.50	10.21	1.09

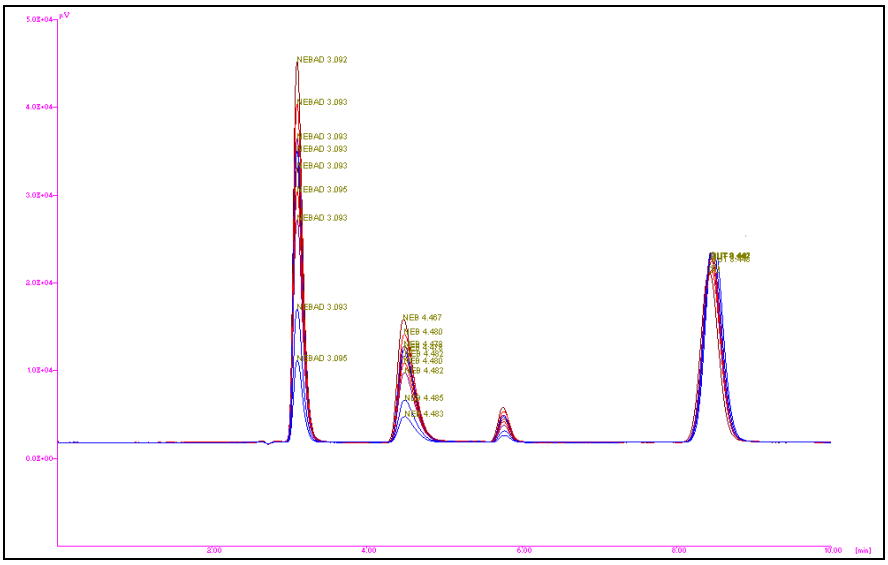
NEBAD- Degradant of nebivolol hydrochloride by acid hydrolysis; NEB- Nebivolol hydrochloride; DUT- Dutasteride as internal standard

Table 7: System suitability parameters for oxidation degradation of NEB

Name of peak	R.T.	Theoretical plates	Selectivity	Capacity	Resolution	Asymmetry
NEBO	2.91	276.68	0.00	274.67	0.00	1.06
NEB	4.51	2244.49	1.61	441.83	5.78	1.62
DUT	8.04	6496.09	1.82	803.67	9.38	1.10

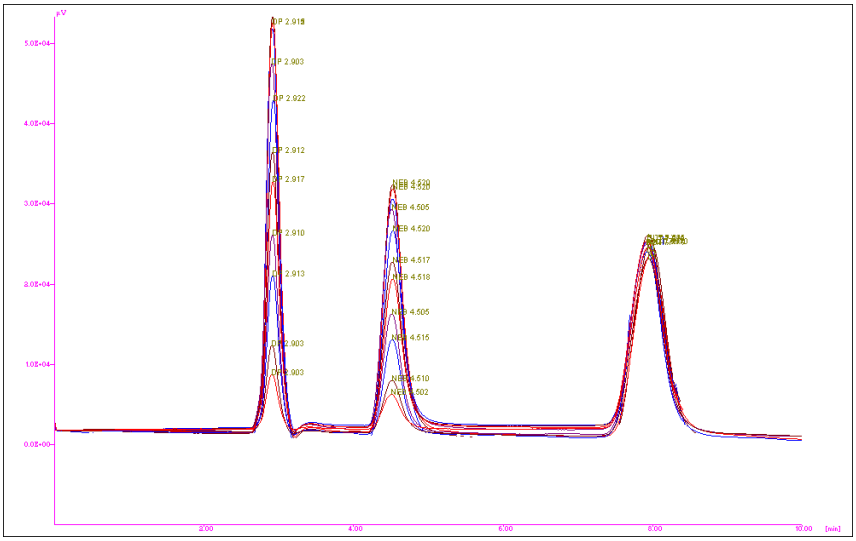
NEBO- Degradant of nebivolol hydrochloride by oxidation; NEB- Nebivolol hydrochloride; DUT- Dutasteride as internal standard

Figure 1: Chromatogram showing recovery study of NEBAD



NEBAD- Degradant of NEB by acid hydrolysis (RT 3.09±0.07 min), NEB- Nebivolol hydrochloride (RT 4.47±0.06 min); DUT- Dutasteride as internal standard (RT 8.47±0.04 min)

Figure 2: Chromatogram showing recovery study of NEBO



NEBO- Degradant of nebivolol hydrochloride by oxidation (RT 2.91±0.04 min); NEB- Nebivolol hydrochloride (RT 4.51±0.06min); DUT- Dutasteride as internal standard (RT 8.04±0.05 min)

Synthesis, isolation and characterization of degradation products

The NEBAD was synthesised in sufficient quantities by acid hydrolysis of NEB. Hydrolysis was carried out using 5 ml of 0.1N HCl per milligram of NEB. The solution was refluxed at 70^0C for 1 hr. After cooling at room temperature, it was neutralised. The isolation of NEBAD as impurity was done by using column chromatography. The oxidative NEBO impurity was also synthesised in 30% H_2O_2. The reaction mixture was stored at room temperature for 24 h. The NEBO was isolated by using column chromatography. The synthesised impurities were characterised by

using IR, NMR and mass spectras. The IR shows values as 3345 (NH stretching); 3215 (OH stretching); 2950 (CH stretching); 1310 (CN stretching), 1100 (C-O stretching Cyclic ether) and 3345 (NH stretching); 2950 (CH stretching); 1710 (C=O stretching); 1310 (CN stretching), 1100 (C-O stretching Cyclic ether) for NEBAD and NEBO respectively. The NMR spectras appeared at 1.90 (d, 2H, CH2-CH2-CH); 2.0 (s, 1H, OH). 2.02 (s, 2H, NH2); 2.45 (s, 3H, HN-CH3); 2.55 (d, 2H, CH2-CH2-CH); 2.70 (s, 2H, NH-CH-CHOH); 3.76 (d, 1H, CH2-CH-O); 3.95 (t, 1H, CH-OH), 6.7-7.10 (m, 4H, Aromatic) and 2.0 (s, 4H, NH2); 2.28 (t, 4H, CH2-CH2-CH); 2.55 (d, 4H, CH2-CH2-CH); 3.71 (s,4H, CH2-NH-CH2) 4.55 (d, 2H CH2-CH-O); 6.5-7.0 (m, 6H, Aromatic) for NEBAD and NEBO respectively. Mass spectras in the negative electron spray ionization (ESI) mode and probable structures of for the degradants are shown in Figure 3 and Figure 4 for NEBAD and Figure 5 and Figure 6 for NEBO, respectively. The m/z values of the peaks are 206 and 406.2 which matched 1-(3,4-dihydro-2H-chromen-2-yl)-2-(methylamino) ethanol and 2,2'-iminobis[1-(6-fluoro-3,4-dihydro-2H-chromen-2-yl) ethanone] as degradant of NEB by acid hydrolysis and degradant of NEB by oxidation, respectively.

Figure 3: MS-MS spectra showing NEBAD

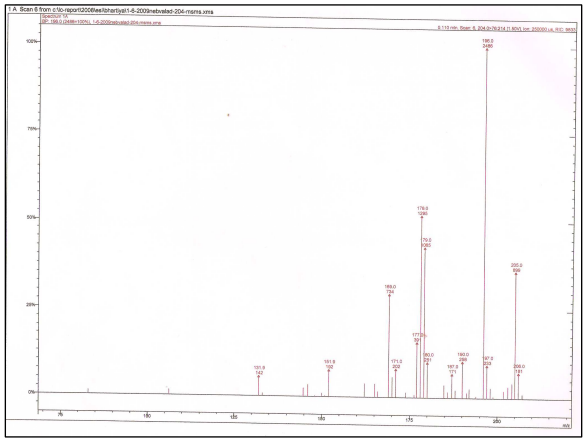


Figure 4: Probable structure of major degradation product(1-(3,4-dihydro-2H-chromen-2-yl)-2-(methylamino)ethanol) of NEB by acid hydrolysis

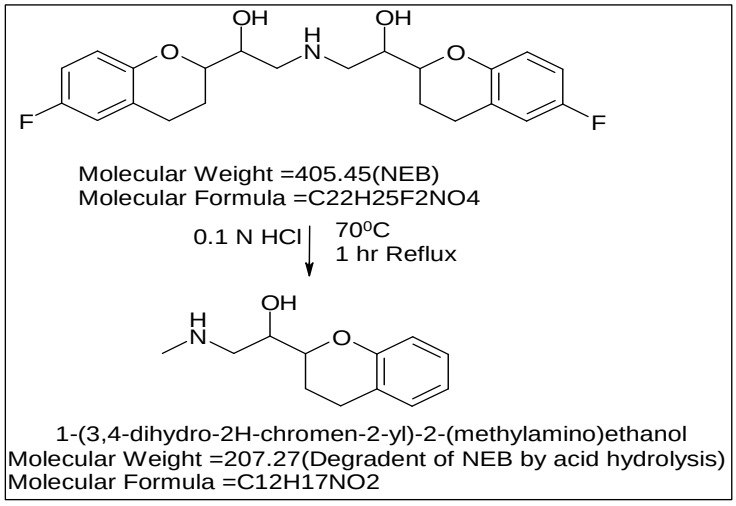


Figure 5: MS-MS spectra showing NEBO

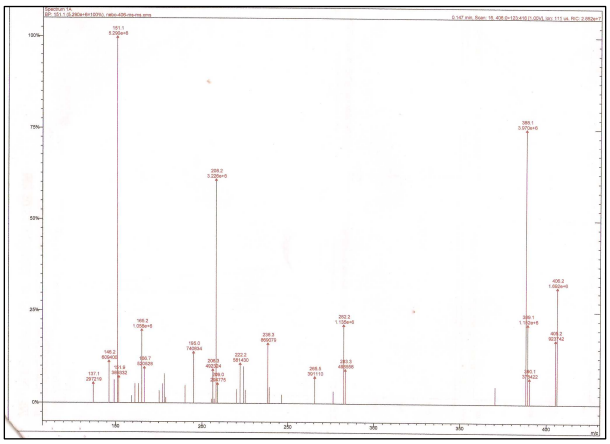
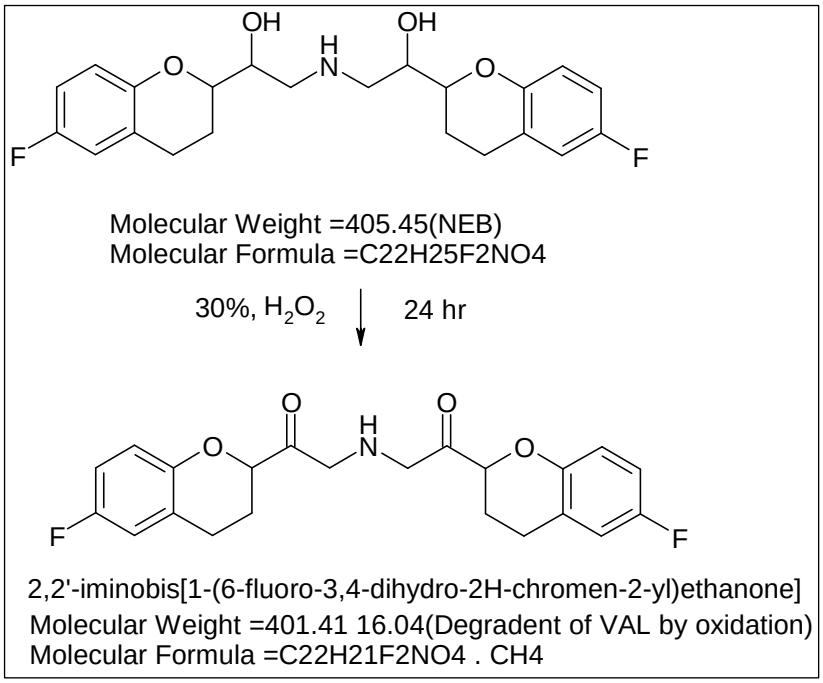


Figure 6: Probable structure of major degradation product(2,2'-iminobis[1-(6-fluoro-3,4-dihydro-2H-chromen-2-yl)ethanone]) of NEB by oxidation



Applicability of the developed method to stability samples

The developed method was found to apply even to real stability samples, which was verified through successful analyses of NEB, which had been stored at accelerated conditions of temperature (40^0C) and humidity (75% RH) for 3 months. Indirectly, it was also established that the developed method could even be used for formulations containing NEB.

Materials and methods

Materials

NEB was supplied as gift sample by Emcure pharmaceuticals ltd. (Pune, Maharashtra, India) and used without further purification. Dutasteride was supplied as gift sample by Cipla ltd. (Mumbai, Maharashtra, India). Sodium hydroxide and hydrochloric acid (both AR grade) were purchased from Loba chemie ltd. Hydrogen peroxide was procured from S.D. fine chem ltd. Methanol (HPLC grade) was purchased from Merck ltd. Double distilled water was obtained from a water distillation unit. Analytical grade triethylamine and glacial acetic acid were purchased from Loba chemie ltd.

Instrumentation

The HPLC system used was a computer based Jasco series instrument comprising of a pump PU-2080 and a UV detector UV-2070. Manual injections were carried out using a Rheodine injector with a fixed 20 µl external loop. The chromatographic separations were performed on a HIQ sil C_{18} ODS column (250 mm length ×4.6 mm internal diameter and 5 µm particle size), operating at ambient temperature, using a mobile phase consisting of methanol:water [80:20v/v, (pH 7.2, adjusted by adding 0.2 M glacial acetic acid into 0.2 M triethyl amine)], at a flow rate of 1.0 ml/min, and detection was performed at 222 nm using a UV detector. HPLC instrument was controlled by software Borwin. The mobile phase was filtered through 45µm nylon membrane filter. A shimadzu AY 120 analytical balance was used for weighing. A PCi ultrasonicator was used for sonication. The calibrated glasswares were used throughout the experiment. The mobile phase was used for dilutions of degradation samples throughout the analysis.

Precision water baths were used for degradation studies under acidic and alkaline conditions. Dry bath was used for thermal stress studies. UV degradation was carried out in a UV chamber equipped with a light bank consisting of two UV and fluorescent lamps. MS studies were carried out in negative electro spray ionization (ESI) mode on Varian Inc, USA., 500 MS IT with 410 Prostar Binary LC (Direct Infusion Mass with ESI and APCI negative and positive mode ionization, mass ranging from 50 to 2000 m/e) instrument. The characterization was done by using FTIR: KBr (cm-1), 4100 JASCO and NMR: DMSO-d6 (δ); 400 MHz Varian NMR.

Conduct of stress studies

The stress studies were carried out under the conditions of dry heat, hydrolysis, oxidation, and UV degradation, as defined by ICH [10]. For thermal stress testing, the drug powder was sealed in glass ampoules and heated in dry bath at 50^0C for 8 hrs. Acid decomposition was carried out in 0.1N HCl at drug strength of 1mg/ml. The studies in alkaline conditions were conducted similarly at a drug concentration of 1mg/ml in 0.1N NaOH. These solutions were refluxed at 70^0C for 1 hr. After cooling at room temperature, the solutions were neutralised separately. The oxidative stress studies were conducted at drug strength of 1mg/ml in 3% H_2O_2. The solution was stored at room temperature for 24 h. As sufficient decomposition was not observed, the drug was additionally exposed at a concentration of 1mg/ml in 30% H_2O_2 at room temperature for up to 24 h. The UV degradation studies were carried out in solid state by spreading a thin layer of drug in a petri-dish and exposing it directly to the combination of UV and florescent light. A parallel set was kept in dark under similar conditions. Samples were withdrawn after 24 hrs.

Standard Preparation

The standard solutions of NEBAD and NEBO containing 1000 µg/ml was prepared in a 100 ml volumetric flask by dissolving 100 mg of each separately. The solutions were sonicated and the final volumes were made with appropriate mobile phase. From these solutions series of dilutions were prepared.

Test Sample Preparation

The test solution for acid decomposition was prepared after cooling reaction solution of drug with acid. The solutions was neutralised and final volume of was made to 100 ml with mobile phase. The sufficient decomposition was observed in 30% H_2O_2 at room temperature for up to 24 h. The oxidative stress test sample was prepared by adjusting final volume to 100 ml with mobile phase.

Separation studies

The reaction solutions were individually subjected to HPLC studies. The studies were conducted using a mobile phase composed of methanol:water [80:20v/v, (pH 7.2, adjusted by adding 0.2 M glacial acetic acid into 0.2 M triethyl amine)]. The separation was achieved by changing the mobile phase composition as well as the flow rate. The overall objective here was to develop a selective stability indicating assay method (SIAM) [11].

Validation of the method

Validation of the optimised HPLC method was done with respect to various parameters, as required under ICH guideline Q2(R1) [12]. To establish linearity and range, a stock solutions of NEBAD and NEBO were prepared separately at strength of 1mg/ml, which were further diluted to prepare solutions in the concentration range of 20-100 µg/ml. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (20 µl). Precision of the method was studied by making six injections of three different concentrations, viz., 30, 60 and 90 µg/ml on the same day and the values of relative standard deviation (% R.S.D.) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision. Accuracy was evaluated by fortifying a mixture of degraded solutions with known concentration of, viz., 20 µg/ml of each NEBAD and NEBO for degradation solution of acid hydrolysis and degradation solution of oxidation, respectively. The recovery of the added impurity was determined. The specificity of the method was established through study of resolution factors of the impurity peak from the nearest resolving peak, and also among all other peaks.

Application of the developed method to stability samples

The developed method was used to analyze stability samples of NEB. The drug was under accelerated conditions of temperature and humidity for 3 months before analysis.

Conclusion

It was possible in this study to develop a stability-indicating HPLC assay method for NEBAD and NEBO by subjecting the drug to ICH recommended stress conditions. The drug and

degradants got well separated from each other in an isocratic mode using a reversed-phase C_{18} column and mobile phase composed of methanol:water [80:20v/v, (pH 7.2, adjusted by adding 0.2 M glacial acetic acid into 0.2 M triethyl amine)]. The flow rate and detection wavelength were 1.0 ml/min and 222 nm, respectively. The method proved to be simple, accurate, precise, specific and selective. It was successfully employed for analysis of the drug and degradants in the marketed products stored for 3 months under accelerated conditions of temperature and humidity. In this study the individual degradants were isolated and characterised. The degradation products were characterized through spectral studies. The stress studies and subsequent spectroscopic analyses showed that the drug was decomposed to degradation products, viz., 1-(3,4-dihydro-2H-chromen-2-yl)-2-(methylamino)ethanol and 2,2'-iminobis[1-(6-fluoro-3,4-dihydro-2H-chromen-2 yl)ethanone] as acid hydrolysis of NEB and oxidation of NEB, respectively.

Acknowledgments

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