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VALIDATED HPLC METHOD FOR DETERMINATION OF NEBIVOLOL IN PHARMACEUTICAL DOSAGE FORM AND IN VITRO DISSOLUTION STUDIES

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ABSTRACT. A rapid and simple liquid chromatographic method was developed and validated according to current ICH guidelines for the quantitative assessment of nebivolol from tablet dosage forms and dissolution medium. Chromatography was carried out on a BDS Hypersil C18 column (150 x 4.0 mm, particle size 5 µm), employing a Merck 7000 series HPLC system with UV detection at 281 nm. The mobile phase consisted of 0.1 % (v/v) trifluoroacetic acid in water: acetonitrile (60:40, v/v) and was delivered at a flow rate of 1.25 mL min^{-1}. Injection volume was 100 µL and the analysis was performed at ambient temperature. The developed method was validated taking into consideration current international guidelines for specificity, linearity, accuracy, precision (system precision and both intra- and interday precision). The validated analytical method proved to be suitable for quantitative analysis of nebivolol from commercially available tablets and also performed well in determination of active substance during dissolution studies.

Keywords: *nebivolol, dissolution, HPLC*

INTRODUCTION

Nebivolol (NEB), 2,2'-Azanediylbis(1-(6-fluorochroman-2-yl)ethanol) (Fig. 1) is third-generation, highly cardioselective $beta_1$-receptor blocker with a unique haemodynamic profile. It combines beta-adrenoreceptor blocking activity with endothelial L-arginine nitric oxide (NO) pathway mediated vasodilation [1,2]. Apart from its antihypertensive properties, NEB also improves arterial compliance and left ventricular function in heart failure. Owning to its unique

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pharmacodynamic properties, it provides higher response rates and presents lower frequency and severity of adverse event compared with other agents from its class [3,4].

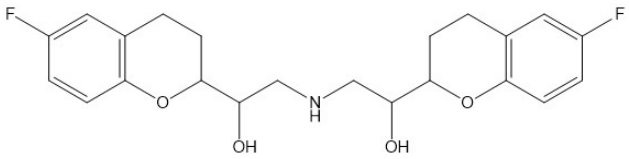

Figure 1. Chemical structure of NEB

Chemically, it differs from other beta-receptor blocker agents, displaying a symmetrical configuration [5]. According to the Biopharmaceutical Drug Classification System, NEB is a class II compound, characterized by low, pH dependent aqueous solubility and high membrane permeability. It is a weakly basic compound, with a pKa of 8.22 [6,7].

Given the therapeutic importance of this beta-blocker and also its intensive use in combination antihypertensive therapy, there are several methods describing the quantitative assessment of NEB from dosage forms, including spectrophotometric[8-10], thin-layer chromatographic [10-12] and liquid chromatographic [9,10,13-16] methods. However, there are no analytical methods described for the chromatographic analysis of NEB from dissolution samples. Moreover, according to the authors' knowledge, full dissolution profiles of NEB and subsequent dissolution behaviour analysis has not been published yet.

Our aim was to develop a simple, rapid and efficient high-performance liquid chromatographic (HPLC) method suitable for the quantitative assessment of NEB from both pharmaceutical dosage forms and dissolution media, which could be applied in routine quality control. In order to meet regulatory requirements, validation of the method, according to current ICH Guidelines was prime ordinary. Investigation of dissolution behaviour for NEB was also targeted, employing three different dissolution media and applying different experimental conditions.

RESULTS AND DISCUSSION

Method development and validation

Among the methods used for quality control of pharmaceuticals, liquid chromatographic methods excel with higher specificity and selectivity. In order to accurately quantify NEB, reverse-phase high-performance liquid chromatography was chosen as the method of choice. Our aim was to

establish a high-throughput method, suitable for rapid quantification of the active substance from a large number of dissolution samples occurring during dissolution profile assessment. The method should also be selective, in order to unequivocally determine the analyte in the presence of possible interfering species (*i.e.* excipients in the case of dosage form).

Several mobile phase compositions were employed, using methanol or acetonitrile as organic modifiers and aqueous solutions of acetic acid and trifluoroacetic acid. In order to achieve adequate retention of NEB, but also maintain a short analysis time, a mobile phase consisting of 0.1 % (v/v) trifluoroacetic acid in water and acetonitrile in a proportion of 60:40 (v/v) was chosen. Using base-deactivated silica stationary phase (BDS Hypersil C18) over "classical" C18 column, an improvement of peak shape was observed for the basic analyte. Using the abovementioned conditions and employing a flowrate of 1.25 mL min^{-1}, we succeeded in eluting NEB in under 4 minutes at ambient temperature.

Validation was carried out in accordance to current ICH Validation Guideline [17], in terms of specificity, linearity, accuracy and precision (system precision, repeatability and intermediate precision).

In order to test the specificity of the method, chromatograms of standard and sample solutions were recorded and dissolution media were also injected (Fig. 2). No interference was observed at the retention time of NEB, moreover both standard and sample solutions showed comparably high peak purity results (>0.98); results indicating that the method could determine the analyte in the presence of potentially interfering species.

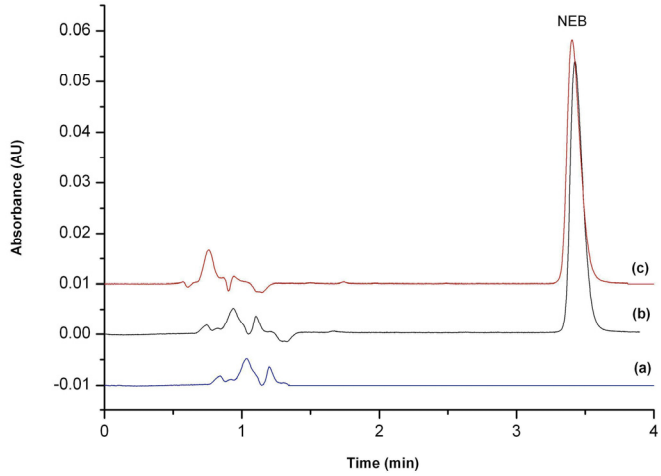

Figure 2. Representative chromatograms of: (a) dissolution medium/solvent (HCl 0.1 N), (b) tablet sample solution, (c) standard solution.

The method performed well during validation, with all results being within the acceptance limits (Table 1), thus demonstrating its suitability for its intended purpose.

Table 1. Summary of method validation results

Studied parameter	Results	
Linearity (N=15, n=5)	Range ($\mu g\ mL^{-1}$) Slope (a) Intercept (b) r^2	1-12 28433 10885 0.997
Accuracy and repeatability (N=9, n=3)	Mean recovery (%) SD RSD (%) CI (95 %)	99.57 1.575 1.582 98.36-100.78
System precision (n=10)	Mean (ASC) SD RSD (%) CI (95 %)	181173.8 791.3 0.437 180606.9-181739.1
Inter-day precision (N=18, n=3)	Mean (%) SD RSD (%) CI (95 %)	99.91 0.616 0.617 99.52-100.30
Analyst variation (N=18, n=3)	Mean (%) SD RSD CI (95 %)	100.26 0.826 0.824 99.74-100.78

N – total number of experiments; n – number of concentration levels;
r^2 – coefficient of correlation; SD – standard deviation; RSD – relative standard deviation

Quantitative determination of NEB from tablets

The validated method was applied for determination of NEB from tablet dosage form. Two separate commercially available products were analyzed, one batch with a valid expiration date and another batch with expired shelf-life. Although, the formulation with expired validity date had a lower NEB content, results indicated that the active substance content of both formulations were between acceptable limits *i.e.* ±5% of the nominal concentration (Table 2).

Table 2. Quantitative determination of NEB from tablets

Sample number	NEB content (mg/tablet)	
	Valid Batch	Expired Batch
1	4.77	4.54
2	4.68	4.63
3	4.75	4.58
Mean	**4.73**	**4.58**
SD	0.047	0.045
RSD (%)	0.998	0.984

In vitro dissolution studies

Proper dissolution testing methodology is essential for evaluating quality of solid pharmaceutical dosage forms. In the case of BCS Class II compounds, such as NEB, presenting low, pH dependent aqueous solubility, the assessment of dissolution profiles at different pH values is not only a regulatory requirement, but also a necessity.

Determination of *in vitro* release profiles of NEB were performed employing three different dissolution media, varying the volume of the medium (500 and 900 mL) and stirring speed (50 and 75 rpm).

NEB, having a weakly basic character, presented high dissolution rates in HCl 0.1 N (Fig. 3a). Under the acidic conditions employed, the secondary amino group from the structure of NEB is fully protonated, thus possessing higher solubility. Using higher dissolution medium, the release profiles of NEB were almost identical, regardless of the rotation speed employed. Whereas, in the case of lower dissolution medium volume, there was a noticeable difference between the first timepoint (67.32 % dissolved at 50 rpm rotation speed versus 91.60 % dissolved at 75 rpm). At later timepoints, these differences started decreasing and at the end of the dissolution test, the full quantity of NEB was dissolved in all cases.

At pH 4.5, dissolution rate of NEB decreased (Fig. 3b). Still, employing high dissolution medium volume and high rotation speeds, almost 80 % of NEB was dissolved at 10 min. However, when the dissolution volume of 500 mL was utilized, along with mild agitation conditions (50 rpm) the slowest dissolution rate was recorded. Nonetheless, at the final sampling point, all dissolution efficacies were above 90 %, in all cases.

Dissolution kinetics of NEB was the slowest at pH 6.8: with an increase of pH, as ionization decreased, solubility also decreased, resulting in poor dissolution efficacy (Fig. 3c). In order to achieve a final cumulative dissolution of above 80 %, 900 mL of dissolution volume needed to be

used, along with a higher rotation speed (75 rpm). Whereas, employing the "harshest" conditions (500 mL, 50 rpm), at the end of the test only 52.12 % of NEB was dissolved.

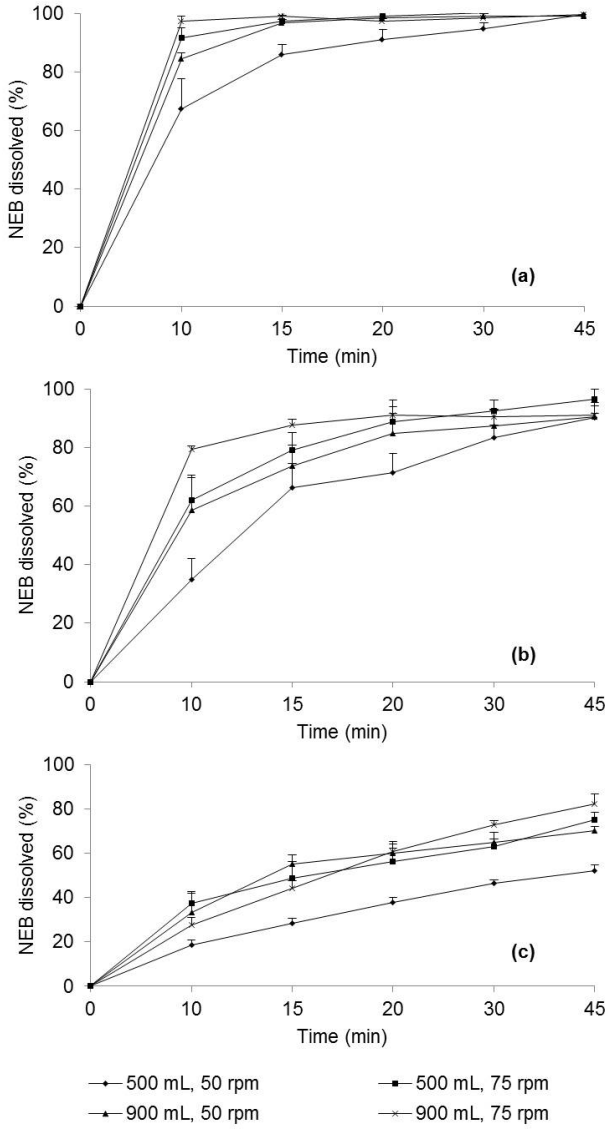

Figure 3. Dissolution profiles of NEB in (a) HCl 0.1 N, (b) acetate buffer pH 4.5, (c) phosphate buffer pH 6.8, employing different conditions. (error bars represent standard deviation values)

CONCLUSIONS

An efficient reverse-phase HPLC method was developed for the determination of NEB from tablet dosage form and dissolution samples. Use of a simple, easy to prepare mobile phase, combined with short analysis time, make the method a good contender for routine quality control testing. The method performed well during validation studies and was subsequently applied for comparative quantification of NEB from valid and expired tablets. Dissolution profiles of NEB were also constructed, employing three different dissolution media and various experimental conditions (different volume of dissolution medium and rotation speed). As expected, results revealed the pH dependent dissolution of NEB and a great influence of the instrumental variables upon dissolution efficacy.

EXPERIMENTAL SECTION

Reagents

Nebivolol clorhidrate working standard was obtained as a free sample from Nivika Chemo Pharma PvT, India. Supergradient grade acetonitrile, acetic acid (glacial), hydrochloric acid (solution, 35 %) were from Merck KGaA (Germany). Sodium acetate trihydrate, sodium hydroxide and methanol were from Lach Ner (Czech Republic), while trifluoroacetic acid and potassium dihydrogen phosphate were from Chemical (Romania). All reagents were of analytical grade, purchased through a local vendor and used without further purification.

Ultrapure, deionized water was prepared with a Millipore Direct Q5 water purification system (Merck Millipore, Germany) and was utilized for chromatographic purposes.

Apparatus

The HPLC system was a Merck Hitachi LaChrom Series 7000, equipped with a quaternary L-7100 pump, L-7200 autosampler, L-7360 column thermostate, L-7455 diode-array detector and L-7612 degasser. Data acquisition was performed using D-700 HSM Manager software. Chromatographic separation was carried out on a Hypersil^{TM} BDS C18 column, with dimensions of 150 x 4.6 mm, particle size 5 µm (Thermo Scientific, USA) at ambient temperatures. Final mobile phase consisted of 0.1 % (v/v) triflouroacetic acid in water: acetonitril 60:40 (v/v), delivered at a flowrate of 1.25 mL min^{-1}. Injection volume was 100 µL and detection was performed at 281 nm.

Dissolution studies were performed using Apparatus 2 (paddle) setting on an Erweka DT 80 dissolution tester, coupled with an ET 1500l heater/circulator, maintaining the temperature of the dissolution medium at

37.0 ± 0.5 °C. Three different dissolution media (HCl 0.1 N, acetate buffer pH 4.5 and phosphate buffer pH 6.8) were tested at two different volumes (500 mL and 900 mL) and two different rotation speeds (50 and 75 rpm). Samples of 3 mL were withdrawn at 10, 15, 20, 30 and 45 min and filtered through a 0.45 µm polyamide filter. The withdrawn volume of sample was replaced with an equal volume of preheated medium.

Preparation of solutions

NEB stock solution (concentration: 0.2 mg mL^{-1}) was prepared by dissolving 21.8 mg NEB hydrochloride (equivalent to 20 mg NEB) in methanol and diluting it to 100 mL. Appropriate dilutions were made from this solution with HCl 0.1 N for validation studies.

Standard solution for tablet assay: 1 mL NEB stock solution was diluted to 50 mL with HCl 0.1 N (concentration: 4 µg mL^{-1}).

Standard solution for dissolution studies were prepared the same way as for tablet assay, weighting appropiate ammount of NEB hydrochloride to match 100 % of dissolution concentrations (aprox. 5.56 µg mL^{-1} for 900 mL dissolution medium and 10 µg mL^{-1} for 500 mL, respectively).

Tablet sample solution: appropiate quantity of tablet powder was weighted, ultrasonicated in 50 mL methanol for 20 minutes and diluted to 100 mL with the same solvent. 1 mL of the obtained solution was diluted to 50 mL with HCl 0.1 N.

Validation of the method

In order to test specificity, the chromatograms of standard, sample solutions and dissolution samples were recorded and compared.

Linearity was assessed over a concentration range of 1-12 µg mL^{-1}, at five concentration levels with three replicates each.

Accuracy and repeatability was assessed at three different concentrations (1, 5.56 and 12 µg mL^{-1}), each concentration being prepared in triplicate. The same concentration levels were also used in order to assess intermediate precision: solutions were prepared on different days by the same analyst (inter-day precision) or were prepared by a different analyst, on the same day (analyst variation). System precision was evaluated by injecting the same solution (5.56 µg mL^{-1}) 10 times.

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