Original Article

Quantification of nebivolol hydrochloride in human plasma by liquid chromatography using fluorescence detection: Use in pharmacokinetic study

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ABSTRACT: A simple, rapid, and sensitive method of reversed-phase high-performance liquid chromatography with fluorescence detection has been developed and validated for use in determining levels of nebivolol·HCl in human plasma. Sample preparation involves a simple single-step protein precipitation procedure and extraction of nebivolol in acetonitrile. The separation was performed on a Kromasil® RP-C18 column (Φ 4.6 mm × 250 mm, 5 µm) with a mobile phase consisting of 0.05 M potassium dihydrogen phosphate buffer/acetonitrile (40:60, v/v) adjusted to pH 3 using orthophosphoric acid. Analysis was carried out under isocratic conditions at a flow rate of 1.5 mL/min and at room temperature using a fluorescence detector with excitation at 288 nm and emission at 310 nm. The chromatographic run was 4 min. The calibration curve was linear over the concentration range 0.2-20 ng/mL. The method was validated in terms of its accuracy, precision, and specificity. The assay enabled the measurement of nebivolol with a minimum quantification limit of 0.16 ng/mL. The average recovery of nebivolol from spiked human plasma was 98.4 ± 3.3%. This method was successfully used in a pharmacokinetic study of oral administration of 5-mg tablets to healthy human volunteers.

Keywords: Nebivolol, plasma, high performance liquid chromatography, fluorescence detection, pharmacokinetic study

1. Introduction

Chemically, nebivolol·HCl is (1RS,1'RS)-1,1'-[(2RS,2'SR)-bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]-2,2'-iminodiethanol hydrochloride (Figure 1). Nebivolol is approximately 3.5 times more β_1-adrenoceptor-selective than other β_1-adrenergic blockers in human myocardium and thus might be the most β_1-adrenoceptor-selective antagonist available for clinical practice at the moment (*1*). It has a combined vasodilating β_1-blocker activity with a vasodilating effect mediated by the endothelial L-arginine nitric oxide pathway (*2,3*). Nebivolol is a racemic mixture of L-nebivolol (RSSS) and D-nebivolol (SRRR) present in equal proportions, as both are necessary for the drug to have maximum effect (*4,5*). The effect of nebivolol on heart rate is exclusively exerted by D-nebivolol (affinity for β_1-receptors that is 100-fold higher than that of the L-isomer). Both the D- and L-isomers facilitate nitric oxide release to induce a vasodilator effect (*6,7*).

Several methods for determining levels of nebivolol in pharmaceutical dosage forms have been described, including UV spectrophotometry (*8*), capillary electrophoresis (*9*), high-performance thin-liquid chromatography (HPTLC) (*10*), and high-performance liquid chromatography (HPLC) (*10,11*) with UV detection. Two chromatographic methods focused on the separation of enantiomers (*12*) and the determination of nebivolol metabolites (*13*). Few methods for determining the level of nebivolol in human plasma or serum have been reported, and all depend on mass detection (*14-16*). Although they are sensitive and have low quantitation limits, mass detection has its limitations. The equipment is complex and requires extensive technical expertise for its

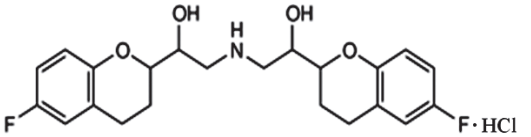

Figure 1. Chemical structure of nebivolol hydrochloride.

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maintenance and operation. Moreover, it is not affordable and not easily available to most research laboratories because of the high cost (*17,18*). Only one of these methods has been used in a study of the pharmacokinetics of fixed oral dosage forms combining nebivolol and valsartan. Although one method of determining the level of nebivolol in plasma based on fluorescence detection was previously developed (*19*), it suffered from the disadvantage of lengthy and highly complicated sample pretreatment. The extraction method was time-consuming due to multiple-step procedures. With the reported method, nebivolol was extracted from alkalinized human plasma with heptane/isoamyl alcohol (95:5, v/v), back-extracted with dilute sulfuric acid, and re-extracted after alkalinization. These multiple steps compromised the accuracy and sensitivity of the method.

This paper describes the development and validation of a simple, rapid, and sensitive method of isocratic reversed-phase HPLC for determining the level of nebivolol in human plasma using fluorescence detection. The method described here is an approach to simplify the sample preparation procedures while maintaining a high level of sensitivity and specificity and minimizing the operating cost. Sample preparation involves simple single-step protein precipitation. In addition, the use of a small sample volume and short analysis time provides advantages for analyzing nebivolol in plasma. The method was successfully used in a pharmacokinetic study of humans.

2. Materials and Methods

2.1. *Chemicals*

Nebivolol•HCl was generously supplied by Berlin Chemie AG, Berlin, Germany. HPLC-grade water, acetonitrile, and methanol were obtained from Sigma GmbH, Mannheim, Germany. Orthophosphoric acid and potassium dihydrogen phosphate were obtained from Ridel-de Haën, Germany. Nebilet® tablets produced by Berlin Chemie AG, batch No. 74529, labeled to contain 5 mg of nebivolol•HCl, were obtained from a local market.

2.2. *Equipment and chromatographic conditions*

The chromatographic apparatus consisted of an HPLC system (Young Lin, Anyang, Korea) equipped with a gradient pump (HPLC pump SP 930D), Young Lin Acme 9000 vacuum degasser & mixer, and LC305 fluorescence detector. Data acquisition was performed with Autochrom-3000 software, version 2.0.0. Separation was performed on a Kromasil® RP-C18 column (Φ 4.6 mm × 250 mm, 5 µm; Eka Chemicals AB, Bohus, Sweden). The mobile phase used was acetonitrile/50 mM phosphate buffer, pH 3, adjusted using orthophosphoric acid (40:60, v/v). All analyses were carried out under isocratic conditions at a flow rate of 1.5 mL/min and at room temperature using a fluorescence detector with excitation at 288 nm and emission at 310 nm. The mobile phase was prepared daily and degassed by ultrasonication. All solvents were filtered through a 0.45-µm disposable membrane filter immediately before use.

2.3. *Standard solutions*

A stock solution (100 µg/mL) of nebivolol•HCl was prepared in methanol. Then, 10 µg/mL and 100 ng/mL working standards were prepared in the mobile phase.

2.4. *Sample preparation*

One mL acetonitrile was added to 1 mL of spiked human plasma. The mixture was vortexed for 2 min and centrifuged for 10 min at 8,000 rpm. The upper layer was filtered through a 0.45-µm Millipore syringe filter. Twenty µL of the supernatant were injected onto the liquid chromatograph for analysis.

2.5. *Plasma standard curve*

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at –20°C. After thawing, 1-mL blank (drug-free) plasma samples were spiked with nebivolol working standard solutions to provide concentrations of 0.2, 0.5, 1, 1.5, 2, 3, 4, 10, 15, and 20 ng/mL. The samples were then prepared for analysis as described above.

2.6. *Selectivity and specificity*

Control human plasma, obtained from 12 healthy volunteers, was assessed by the procedure described above and compared with respective plasma samples to evaluate the selectivity of the method.

2.7. *Precision and accuracy*

The precision and accuracy of the method were examined by adding known amounts of nebivolol•HCl to pool plasma. For intraday precision and accuracy, three samples at each concentration were assayed on the same day. The interday precision and accuracy were evaluated on three different days.

2.8. *Limit of quantification (LOQ) and recovery*

The limit of quantification (LOQ) is the analyte concentration producing a signal-to-noise (S/N) ratio ≥ 10. For spiked plasma samples, the relative analytical recovery at three different concentrations of nebivolol (0.5, 4, and 10 ng/mL) was determined by comparing the peak areas for nebivolol extracted from spiked plasma and a standard solution of nebivolol in acetonitrile with the same initial concentration (three

samples for each concentration level).

2.9. *Biological samples*

Twelve healthy male volunteers were included in this study. The study was conducted in accordance with the International Ethical Guidelines for clinical studies in humans set out in the Declaration of Helsinki (*20*) as well as with the latest guidelines on Good Clinical Practice of the International Conference on Harmonization (*21*). Written informed consent was obtained from the volunteers. Nebivolol•HCl was administered to volunteers in a single dose of a 5-mg tablet after fasting overnight. Fourteen blood samples were collected at 0, 15, 30, and 45 min, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12, and 24 h after dosing. The collected samples were immediately centrifuged and the plasma was frozen and stored at –20°C until analysis. Plasma samples were labeled by protocol number, the subject's initials, treatment, and sample time, and samples were then forwarded to the laboratory.

3. Results and Discussion

3.1. *Method development*

A protein precipitation technique was used as an extraction method for sample preparation in this work. A protein precipitation technique can help produce a spectrometrically clean sample and avoid interference with the analyte by endogenous substances in plasma. The mobile phase at different concentrations of acetonitrile was evaluated as a precipitating solvent, but pure acetonitrile was found to be optimal. It produced clean chromatograms for blank plasma samples and yielded the highest recovery for the analyte from human plasma (data not shown).

The chromatographic conditions, including the detector settings, were optimized through several trials to achieve good resolution and symmetric peaks of the analyte as well as a short run time. The effect of the acetonitrile concentration in the mobile phase on the separation of nebivolol was studied. As the concentration of acetonitrile increased, the retention of nebivolol decreased (data not shown). The optimum acetonitrile concentration was found to be 30-45%, as it produced an acceptably short retention time (data not shown). In addition, the effect of buffer pH was investigated. Orthophosphoric acid was found to be necessary in order to lower the pH to protonate the nebivolol and thus deliver a good peak (data not shown). The buffer was adjusted to pH 3. At a higher pH, nebivolol was carried out faster with the mobile phase, but higher un-ionized species led the drug to stick out or partition with the stationary phase (data not shown). This resulted in tailing and hence increased asymmetry of the peak. At a lower pH, the ionized hydrophilic species were not substantially partitioned with the stationary phase and hence produced a symmetric peak (data not shown). Satisfactory results were obtained at a pH between 3.5 and 2.5. The use of a pH lower than 2.5 was not recommended to avoid deterioration of the column. Thus, the best chromatographic separation was achieved at pH 3. The average retention time ± S.D., obtained for six replicates, was found to be 1.81 ± 0.04 min (data not shown). The run time was 4 min, which offers the advantage of rapid analysis and reduction of the consumed solvents.

3.2. *Method validation*

The linearity of the calibration curve was determined by plotting the average peak area versus the nominal concentration of nebivolol•HCl. To evaluate the linearity of the method, plasma calibration curves were determined in triplicate on three different days. Good linearity was observed over the concentration range 0.2-20 ng/mL and the correlation coefficient was 0.9999. No significant changes were observed in the values of the slope, intercept, and correlation coefficient on both interday and intraday calibrations. The lower limit of quantitation of nebivolol using the described method was found to be 0.16 ng/mL (producing a signal-to-noise (S/N) ratio $\geq$ 10). This was sensitive enough for drug monitoring in pharmacokinetic studies. The instrumental response sensitivity is the slope of the calibration line because a method with a large slope is better able to discriminate between small differences in analyte content (*22*). The relative standard deviation (R.S.D.) values for the recovery of nebivolol at three different concentrations were 3.04 and 3.87% for intraday and interday precision, respectively. According to the Washington Conference Report (*23*), the maximum R.S.D. for a method using plasma should be $\leq \pm 15\%$ (and at the limit of quantitation $\leq \pm 20\%$). This indicates that the proposed method is highly precise. The results of assay validation are summarized in Table 1. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves. The average recovery of nebivolol from spiked plasma samples was 98.4 ± 3.3% (n = 3). Table 2 shows the average recovery of nebivolol from spiked plasma samples at different concentrations. Table 3 shows the results of interday and intraday precision.

Several validation documents (*23,24*) require only six different sources of blank matrices to be analyzed to demonstrate that there is no interference in the chromatographic region of the analyte. The proposed method was tested for specificity by comparing chromatograms of 12 different sources of blank human plasma (from 12 volunteers). The chromatograms were free from any interfering peaks at the retention time of nebivolol•HCl. Thus, the proposed method can be used to determine the level of nebivolol•HCl in plasma without interference by endogenous plasma components. A typical chromatogram of human plasma

Table 1. Results of assay validation of the proposed HPLC method for determination of nebivolol•HCl in human plasma

Parameters	Values
Linearity range (ng/mL)	0.2 to 20
Slope	1,326
Intercept	52.78
Accuracy	98.4 ± 3.3%
Correlation coefficient (r^2)	0.9999
Intraday precision (R.S.D. %)*	3.04
Interday precision (R.S.D. %)*	3.87

* The intraday and the interday relative standard deviations were determined using samples with a concentration of 0.5, 4, and 10 ng/mL performed in triplicate.

Table 2. Determination of human plasma samples spiked with nebivolol•HCl using the proposed HPLC method

Sampled (ng/mL)	Found* (ng/mL)	% Recovery (mean ± S.D.)
0.2	0.186	92.9 ± 5.9
0.5	0.477	95.4 ± 2.9
1	0.957	95.7 ± 1.0
1.5	1.45	96.8 ± 1.9
2	1.97	98.4 ± 6.0
3	3.05	101.5 ± 3.6
4	4.05	101.2 ± 4.2
10	10.22	102.2 ± 2.8
15	14.70	88.0 ± 1.3
20	20.35	101.7 ± 4.7
Mean ± R.S.D.		98.4 ± 3.3

* Mean of three determinations.

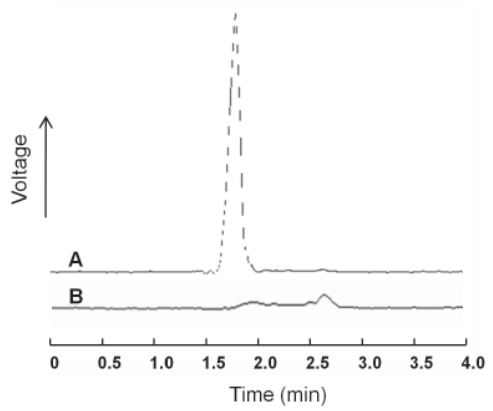

Figure 2. Typical HPLC chromatograms of human plasma. A, human plasma spiked with nebivolol•HCl; **B**, blank drug-free human plasma.

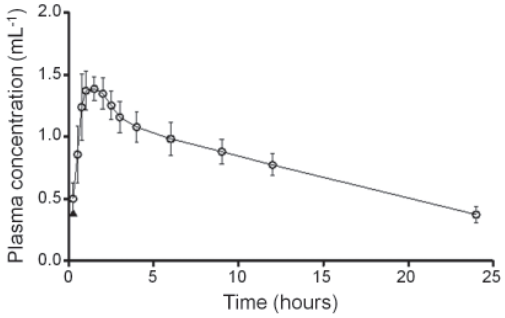

Figure 3. Plasma concentration time curve for nebivolol following oral administration of Nebilet® tablets.

Table 3. Reproducibility of the analysis of nebivolol in human plasma

Concentration added (ng/mL)	Recovery (mean ± S.D.)*	
	Intraday	Interday
0.5	0.48 ± 0.02	0.47 ± 0.02
4	4.01 ± 0.13	4.10 ± 0.20
10	10.22 ± 0.29	10.24 ± 0.34

* $n = 3$.

Table 4. Summary of pharmacokinetic parameters of nebivolol•HCl following oral administration of Nebilet®

Pharmacokinetic parameters	Values (mean ± S.D.)
C_{max} (ng/mL)	1.52 ± 0.09
T_{max} (h)	1.17 ± 0.47
$AUC_{0\text{-}24}$ (ng/mL•h)	18.84 ± 1.92
$AUC_{0\text{-}\infty}$ (ng/mL•h)	25.75 ± 3.42
$K_{elimination}$ (h^{-1})	0.056 ± 0.005
$t_{1/2\ elimination}$ (h)	12.63 ± 1.11

spiked with nebivolol•HCl is shown in Figure 2A, and a chromatogram of blank drug-free human plasma is shown in Figure 2B. The spiked human plasma samples, stored at −20°C and injected over a period of 1 month, did not exhibit any appreciable changes in assay values and were able to meet the criterion mentioned above (data not shown). Nebivolol was also found to be stable in human plasma for more than a month and its stability was maintained at room temperature for more than 12 h. Extraction efficiency was verified by the accuracy of the proposed method and there were no significant differences in the recovery from extracted and non-extracted samples containing the same concentration of nebivolol•HCl (data not shown).

The developed method was successfully used in a pharmacokinetics study of 12 healthy male volunteers who took a 5-mg oral tablet of nebivolol after fasting overnight. Figure 3 shows the mean plasma concentration-time curve for nebivolol•HCl. The plasma concentration reached a maximum 1.17 ± 0.47 h after dosing with a level of 1.52 ± 0.09 ng/mL. The derived pharmacokinetic parameters of the 12 healthy volunteers are summarized in Table 4. These pharmacokinetic parameters are in good agreement with those noted previously (*16,25,26*).

4. Conclusion

A rapid, sensitive, and accurate method of liquid chromatography with fluorescence detection was developed to determine the level of nebivolol•HCl in human plasma. The method has a high sensitivity with a LOQ of 0.16 ng/mL, good linearity in a concentration

range of 0.2-20 ng/mL, and good specificity without interference from endogenous substances in plasma. The method has additional advantages such as a short run time (4 min), low sample volume (20 μL), and simplicity of sample preparation (single-step protein precipitation). These advantages, combined with the relatively inexpensive and readily available equipment used, led to its successful use in a pharmacokinetic study of nebivolol•HCl in human volunteers.

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