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An indirect stereoselective analysis of nebivolol glucuronides in plasma by LC–MS/MS: Application to clinical pharmacokinetics

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ABSTRACT

Nebivolol is a racemate of the *d*-isomer responsible for β_1 adrenergic receptor antagonism and the *l*-isomer responsible for the release of nitric oxide from endothelial cells. Nebivolol is mainly metabolized to nebivolol glucuronide, which also contribute to the nebivolol β_1 adrenoreceptor antagonism. This study reports the development and validation of an indirect stereoselective method of analysis of nebivolol glucuronides in plasma by LC–MS/MS. The method was applied to the investigation of stereoselectivity in the glucuronidation of nebivolol in elderly hypertensive patients ($n = 11$) CYP2D6 phenotyped as EM and treated with a single oral dose of the racemate. One-milliliter plasma aliquots spiked with internal standard (S)-(−)-metoprolol were incubated with 25 μL of β-glucuronidase (final concentration 2500 unit/mL) at pH 5.0 for 16 h at 37 °C. Linearity for total nebivolol was 0.2–125 ng of each isomer per mL plasma and permitted analysis of nebivolol glucuronide isomers up to 48 h after administration of a single oral dose of 10 mg racemate. Regarding to the nebivolol glucuronide isomers, higher plasma concentrations of the *d*-isomer were observed compared to the *l*-isomer (*d*/*l* AUC = 5.4), explaining at least in part the plasma accumulation of unchanged *l*-nebivolol (*l*/*d* AUC = 1.8). This study also showed metabolic glucuronide nebivolol/unchanged nebivolol ratios of approximately 6.5 for the *l*-isomer (AUC 65.3 *vs* 10.1 ng h/mL) and approximately 62.1 (335.2 *vs* 5.4 ng h/mL) for the *d*-isomer. Considering that *d*-nebivolol glucuronide also contributes for β_1 adrenergic receptor antagonism, future studies regarding PK-PD of nebivolol should evaluate not only plasma concentrations of unchanged nebivolol isomers but also glucuronide nebivolol isomers.

1. Introduction

Nebivolol, a drug with four chiral centers, is available for clinical use as a racemate of the *d*-nebivolol (SRRR) and *l*-nebivolol (RSSS) isomers [1,2]. Nebivolol is used to treat systemic arterial hypertension and congestive heart failure. Nebivolol is a third-generation and highly selective β_1 adrenoreceptor antagonist with vasodilatoy effects mediated by nitric oxide via β_3 receptor agonism [3]. The *d*-isomer is a highly selective β_1 adrenoreceptor antagonist, while *l*-nebivolol promotes the release of nitric oxide from endothelial cells [4–6].

Nebivolol is mainly metabolized by direct glucuronidation and secondarily by aromatic hydroxylation and N-dealkylation. The major metabolites found in plasma are glucuronides in addition to the oxidative N-dealkylated acid. N-dealkylation and hydroxylation depend on the activity of CYP2D6, an enzyme that exhibits genetic polymorphism. Some oxidative hydroxylated conjugates are only found in the plasma of extensive metabolizers (EM). In poor metabolizers (PM), the glucuronidation of unchanged nebivolol is considered a primary metabolic pathway. The hydroxylated and glucuronide metabolites also contribute to the nebivolol β_1 adrenoreceptor antagonism [3,6–8].

Previous clinical trials assessing the nebivolol metabolism both as monotherapy or in combination with CYP2D6 inhibitors have been conducted evaluating only its hydroxylated metabolite, 4-hydroxynebivolol. The plasma concentrations of 4-hydroxynebivolol evaluated as isomeric mixture are lower than those of unchanged nebivolol, with nebivolol/4-hydroxynebivolol

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metabolic ratios close to 2. Treatment of healthy volunteers with CYP2D6 inhibitors (paroxetine or bupropion) for 7 days results in an approximately six-fold increase in the area under the plasma concentration versus time curve (AUC) for both the unchanged drug and its 4-hydroxynebivolol metabolite [9–11].

A previous study [12] described the development and validation of an analytical method for measuring unchanged nebivolol isomers in human plasma by LC–MS/MS coupled to a chiral Chirobiotic® V stationary-phase column using a mobile phase of methanol:acetic acid:diethylamine (100:0.15:0.05, v:v:v). In the present study, we describe for the first time an indirect method for the analysis of nebivolol glucuronide isomers in plasma by LC–MS/MS. The nebivolol glucuronide isomers concentrations were calculated as the difference between total nebivolol concentrations and unchanged nebivolol concentrations for each isomer. For the determination of the total nebivolol concentration plasma samples were treated with β-glucuronidase before nebivolol quantitation while unchanged nebivolol was directly quantified. The indirect analytical method for measuring nebivolol glucuronide isomers in plasma was applied to the investigation of stereoselectivity in the metabolism of nebivolol in hypertensive elderly patients treated with a single oral dose of the racemic nebivolol.

2. Experimental

2.1. Analysis of total nebivolol isomers

2.1.1. Chemicals

Racemic nebivolol (98% nebivolol hydrochloride) was obtained from Toronto Research Chemicals (Ontario, Canada). (S)-(−)-metoprolol, used as internal standard (IS), was kindly provided from AstraZeneca (Mölndal, Sweden). HPLC-grade LiChrosolv methanol was purchased from Merck (Darmstadt, Germany). Diethylamine and sodium acetate were obtained from J.T. Baker (Phillipsburg, EUA). β-Glucuronidase (type HP-2 from *Helix pomatia*, glucuronidase activity ≥ 100,000 units per mL and sulfatase activity ≤7500 units per mL) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Water was obtained from Milli-Q system (Millipore, Bedford, MA, USA).

2.1.2. Calibration and quality control samples

The stock solution of racemic nebivolol was prepared at a concentration of 100 μg of the free base per mL methanol. This solution was diluted to obtain working solutions of 8, 20, 80, 200, 400, 1000 and 5000 ng of each nebivolol isomer per mL methanol. One milliliter of blank human plasma samples were spiked with 25 μL of each working solution to obtain nebivolol plasma concentrations of 0.2, 0.5, 2, 5, 10, 25 and 125 ng of each nebivolol isomer per mL plasma.

Quality control (QC) samples were prepared separately, at five different plasma concentrations (0.2, 0.5, 50, 100 and 5000 ng of each nebivolol isomer per mL, respectively as lower limit of quantification (LLOQ), low (LQC), medium (MQC), high (HQC) and dilution (DQC).

Calibration curves included a blank sample (a blank plasma sample processed without the IS), a zero sample (a blank plasma sample processed with the IS) and non-zero samples (0.2, 0.5, 2, 5, 10, 25 and 125 ng per mL). The calibration curves were constructed by plotting the peak area ratios (each nebivolol isomer/IS) versus corresponding concentrations by weighted ($1/x^2$) least-squares linear regression.

2.1.3. Enzymatic hydrolysis and sample preparation

Aliquots of blank human plasma (1 mL) were spiked with 25 μL of (S)-(−)-metoprolol solution (internal standard, 300 ng per mL in methanol), 500 μL of sodium acetate buffer 0.75 M, pH 5.0, and 25 μL of β-glucuronidase (final concentration of 2500 units per mL). The tubes were vortexed for 30 s and the samples were kept in a water bath at 37 °C for 16 h.

After enzymatic hydrolysis, the samples were spiked with 200 μL of a 1 M NaOH solution, 50 mg of NaCl and 4 mL of diisopropyl:dichloromethane (70:30, v:v). The tubes were kept in a horizontal shaker (MA 139/CFT, Marconi, Piracicaba, São Paulo, Brazil) for 40 min. After centrifugation at 2500 rpm for 10 min at 15 °C (Himac CF 8DL, Hitachi, Tokyo, Japan), 3.6 mL of the supernatants were transferred to conical tubes and evaporated to dryness in a vacuum evaporation system (Christ RVC 2-25 CD and Christ CT 04-50 SR, Osterode am Harz, Germany). The residues were resuspended in 200 μL of the mobile phase and 40 μL were used for chromatographic analysis.

2.1.4. LC–MS/MS instrument and conditions

The nebivolol isomers were separated on a Chirobiotic V® column (particle size of 5 μm, 250 mm × 4.6 mm, Astec, Whippany, NJ, USA) maintained at 24 °C. The mobile phase consisted of 95% of a mixture of methanol:acetic acid:diethylamine (100:0.15:0.05, v:v:v) and 5% of a mixture of water:acetic acid:diethylamine (100:0.15:0.05, v:v:v) eluted at a flow rate of 0.8 mL/min.

The mass spectrometry detection system was a Quattro Micro LC triple quadrupole spectrometer (Micromass, Manchester, United Kingdom) equipped with an electrospray interface (ESI). The analyses were carried out in the positive ion mode. The capillary voltage in ESI was 3 kV. The source and desolvation temperatures were maintained at 150 and 550 °C, respectively. Nitrogen was used as the nebulization gas at a flow rate of 150 L/h. Argon was used as the collision gas at a pressure of approximately 3×10^{-4} mbar. The cone voltage was maintained at 40 V for nebivolol and at 25 V for the internal standard (S)-(−)-metoprolol. The collision energy was 30 eV for nebivolol and 15 eV for the internal standard (S)-(−)-metoprolol.

The optimal conditions of tandem mass spectrometry (MS/MS) were determined by direct infusion of the standard solutions of nebivolol (170 ng per mL methanol) and (S)-(−)-metoprolol (internal standard, 250 ng per mL methanol) prepared in the mobile phase and injected with an infusion pump at a flow rate of 10 μL/min. The analysis was carried out in the multiple reaction monitoring (MRM) mode. The protonated ions $[M+H]^+$ and their respective product ions were monitored at transitions of 406 > 151 for nebivolol and of 268 > 116 for (S)-(−)-metoprolol. The elution order and racemization test of the nebivolol isomers had been determined in a previous study [12].

2.2. Analysis of unchanged nebivolol isomers

The analysis of unchanged nebivolol isomers in plasma using LC–MS/MS was conducted as previously described [12] with some modifications. Briefly, plasma aliquots of 500 μL were spiked with 25 μL of (S)-(−)-metoprolol solution (internal standard, 600 pg per mL in methanol), 25 μL of an aqueous solution of 1 M NaOH, 50 mg of NaCl and 4 mL of diisopropyl:dichloromethane (70:30, v:v). The plasma samples were extracted according to Section 2.1.3 and the nebivolol isomers were separated on a chiral column as previously described in Section 2.1.4.

The analyses were carried out on an ACQUITY UPLC® System coupled to a Xevo TQ-S Triple Quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization interface (ESI). Analysis was performed in the positive ion mode. The capillary voltage in ESI was 2 kV. The source and desolvation temperatures were maintained at 150 and 550 °C, respectively. Nitrogen was used as the nebulization gas at a flow rate of 150 L/h. Argon was used as the collision gas at a pressure of approximately 7×10^{-4} mbar. The cone voltage and collision