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Stereoselective analysis of nebivolol isomers in human plasma by high-performance liquid chromatography–tandem mass spectrometry: Application in pharmacokinetics

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ABSTRACT

Nebivolol is available for clinical use as a racemic mixture. Isomer D-nebivolol (SRRR) is a β_1 adrenergic receptor blocker and its antipode, L-nebivolol (RSSS) is responsible for endothelium-dependent NO liberation. This report describes the development and validation of a method of analysis of nebivolol isomers in human plasma by high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS). Nebivolol isomers were extracted from 2 mL aliquots of plasma spiked with tramadol as internal standard, alkalinized and added of sodium chloride and diisopropyl ether:dichloromethane (70:30, v/v). Nebivolol isomers were resolved on a Chirobiotic® V column using methanol:acetic acid:diethylamine (100:0.15:0.05, v/v/v) as mobile phase. Protonated ion and respective ion product were monitored in transitions 406 > 151 for nebivolol and 264 > 58 for internal standard tramadol. There was no racemization of nebivolol isomers during the procedures of sample preparation and chromatographic analysis and matrix effect was absent. Analysis of nebivolol isomers showed linearity for plasma concentrations of 25–2500 pg/mL of each isomer. The quantification limit was 25 pg of each isomer/mL of plasma. Variation coefficients and inaccuracy calculated in precision and accuracy determinations were lower than 15%. Nebivolol was stable in human plasma after three successive cycles of freezing and thawing, during 4 h at room temperature and after processing during 12 h in the auto sampler at 5 °C showing deviation values lower than 15%. The method was applied in a study of the kinetic disposition of nebivolol in plasma samples collected until 48 h after administration of an oral single dose of 10 mg of racemic nebivolol hydrochloride to a patient with systemic arterial hypertension. The clinical study demonstrated that the nebivolol pharmacokinetics is stereoselective. Isomer L-nebivolol showed higher $AUC^{0-\infty}$ (9.4 ng/h/mL *vs.* 4.7 ng/h/mL) and smaller apparent clearance (Cl/f) (531.8 L/h *vs.* 1304.4 L/h) when compared to antipode D-nebivolol.

1. Introduction

Nebivolol, a third generation beta blocker with antihypertensive activity is available in the clinic as a racemic mixture of isomers D-nebivolol [(SRRR)-nebivolol] and L-nebivolol [(RSSS)-nebivolol] [1]. Isomer D- is a potent, selective β_1 adrenergic blocking agent, while isomer L- shows a vasodilator effect mediated through L-arginine/endothelial NO [2].

The enantioselective pharmacokinetics of nebivolol was first described by Van Peer et al. [3] who employed radioimmunoassay using enantioselective antibodies [4]. The authors reported isomeric AUC L/D ratios of about 2 in hypertensive patients orally treated with the racemic drug. However, enantioselective antibodies do not discriminate nebivolol isomers from their aromatic and alicyclic hydroxylated metabolites. *In vitro* studies show that affinity of D-isomers of hydroxylated metabolites for the β_1-adrenergic receptor is comparable to the one of D-nebivolol [3]. Stereospecific radioimmunoassay was also employed by Himmelmann et al. [5] to determine plasma concentrations of nebivolol from hypertensive patients treated during 4 weeks with daily dosages p.o. of 5 mg racemic drug. They observed that steady state plasma concentrations for both isomers (nebivolol plus hydroxylated metabolites) were approximately double the ones observed

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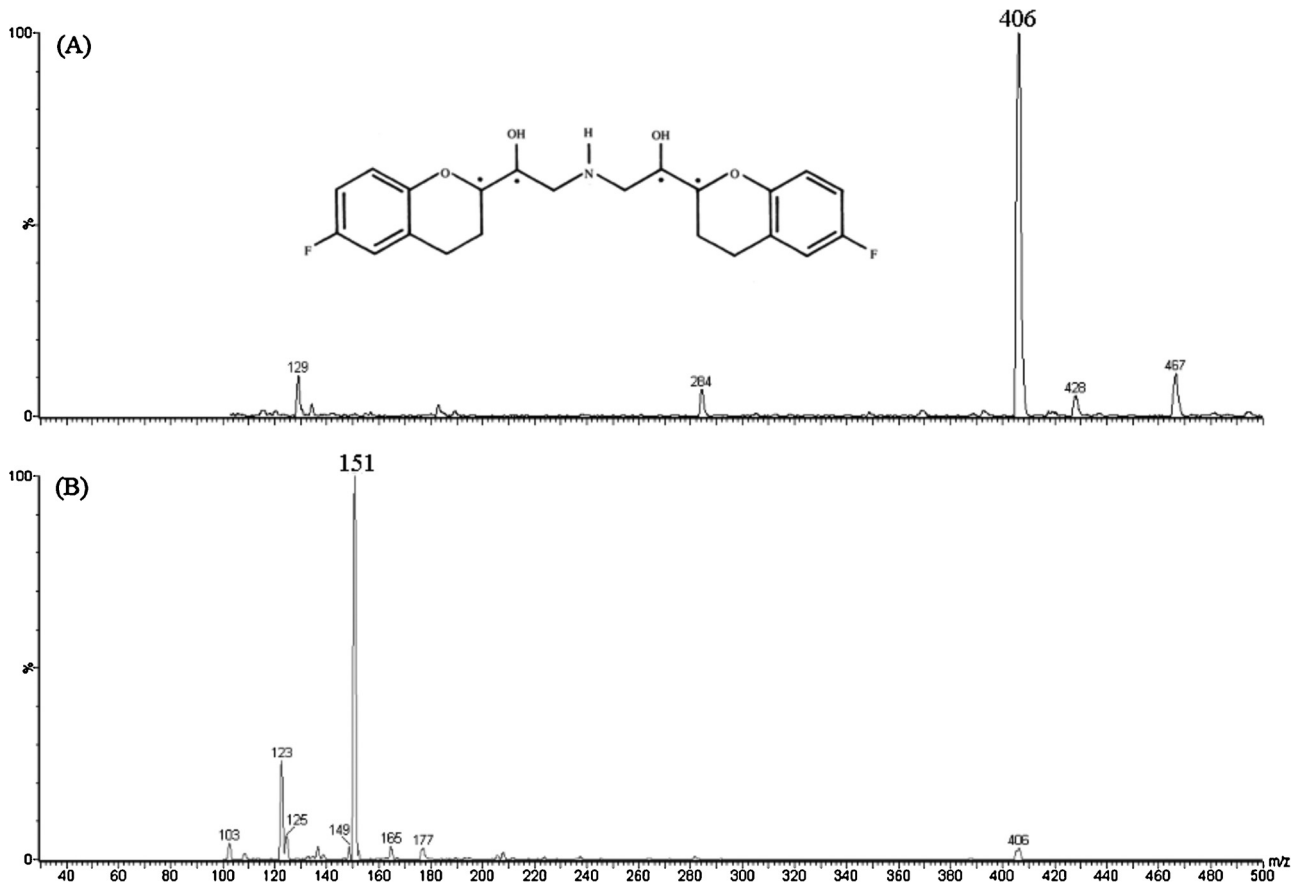

Fig. 1. Full-scan mass spectra of the protonated molecular ion (A) and the ion product (B) of nebivolol.

in the administration of the first dose. The stereospecific radioimmunoassay preceded by an extraction procedure was also utilized by Cheymol et al. [6] to describe the influence of obesity on the pharmacokinetics of nebivolol in patients treated iv with the drug.

High-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) was employed by Lindamood et al. [7] to quantify nebivolol isomers in the range of 20–1500 pg of each isomer/mL of plasma, but without details of the extraction and chromatographic procedures. Their report only shows a figure with plasma concentrations of the individual isomers while pharmacokinetic parameters are described as isomeric mixtures of the drug. Other previous studies had already described the use of LC–MS/MS in the analysis of nebivolol as isomeric mixtures in plasma, employing non chiral columns, in quantification ranges of 10–50 pg of nebivolol/mL of plasma utilizing 200–500 μL of plasma [8–10].

The present study describes the stereoselective analysis of nebivolol in human plasma utilizing a Chirobiotic® V column and a LC–MS/MS detector in quantification limits compatible with pharmacokinetic analysis of single dose administration (quantification limit of 25 pg of each isomer/mL). The validated method was applied in the determination of nebivolol isomers plasma concentrations up to 48 h after the oral administration of a single dose of 10 mg racemic drug to a patient with systemic arterial hypertension.

2. Materials and methods

2.1. Standard solutions and reagents

Racemic nebivolol (nebivolol hydrochloride, 98%) was acquired from Toronto Research Chemical (Ontario, Canada). Racemic tramadol hydrochloride was kindly provided by Janssen-Cilag Farmaceutica (São José dos Campos, São Paulo, Brasil). Extraction solvents, dichloromethane (Philipsburg, NJ, USA) and diisopropyl ether (Tedia Away, Fairfield, OH, USA) and the mobile phase components were obtained from Merck (Darmstadt, Germany) and were of chromatographic grade. Acetic acid (J.T. Baker, Xatoloc, Mexico), diethylamine (J.T. Baker, Phillipsburg, NJ, USA) and sodium hydroxide (Synth, Diadema, SP, Brasil) were of P.A. grade. Water utilized to prepare reagents was from Milli-Q® Plus (Millipore Corp. Bedford, MA, USA).

The stock solution of racemic nebivolol was prepared in the concentration of 100 μg as freebase/mL of methanol. Working solutions were prepared by dilution of stock to the final concentrations of 2, 4, 8, 20, 40, 80 and 200 ng of each nebivolol isomer/mL of methanol. Internal standard (IS) tramadol was prepared as a 50 μg/mL solution in methanol. The working solution, 250 ng/mL in methanol, was prepared by appropriate dilution of stock.

2.2. Chromatographic analysis

The high-performance liquid chromatography (HPLC) was conducted in a Shimadzu instrument (Kyoto, Japan) equipped with a LC-10 pump and CTO-10 AS oven. Isomers were separated in a Chirobiotic® V (Sigma–Aldrich) column (250 mm × 4.6 mm, 5 μm particles) with a mobile phase of methanol: acetic acid:diethylamine (100%:0.15%:0.05%, v/v/v) in an 1 mL/min flux at 24 °C. The mass spectrometry detection system was a Quattro Micro LC triple quadrupole (Micromass, Manchester, UK) equipped with an *electrospray* interface (ESI). Analysis was conducted in the positive ionization mode, the capillary voltage in the ESI was 3 kV and the source and desolvation temperatures were 120 and 200 °C,