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Efficient molecularly imprinted polymer as a pipette-tip solid-phase sorbent for determination of carvedilol enantiomers in human urine

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ABSTRACT

In this work, an efficient pipette tip based on molecularly imprinted polymers solid-phase extraction (PT–MIP–SPE) method was developed for carvedilol (CAR) analysis. This compound is available in clinical practice as a racemic mixture, in which (−)-(*S*)-CAR is a β- and α_1-adrenergic antagonist, while (+)-(*R*)-CAR only acts as an α_1-adrenergic antagonist. Enantioseparation of CAR presented satisfactory retention times (5.85 and 14.84 min), acceptable theoretical plates (N = 2048 and 2018) and good resolution (Rs = 9.27). The separation was performed using a Chiralpak® IA column (100 mm × 4.6 mm, 3 µm), a mixture of methanol:ethanol:water (64:15:21, v/v/v) plus 0.3% diethylamine as mobile phase, temperature of 35 °C and flow rate of 1.5 mL min^{-1}. After density functional theory calculations based on prepolymerization complexes, the best protocol for the MIP synthesis was chosen. Then, some parameters that affect the PT–MIP–SPE technique were investigated. After optimization, the best conditions were 300 µL of water as washing solvent, 500 µL of acetonitrile:acetic acid (7:3, v/v) as eluting solvent, 20 mg of MIP, 500 µL of urine sample (pH 12.5) and no addition of NaCl. Recoveries ± relative standard deviation (RSD%) for (+)-(*R*)-CAR and (−)-(*S*)-CAR were 101.9 ± 4.8% and 104.6 ± 2.1%, respectively. The method was linear over the concentration range from 20 to 1280 ng mL^{-1} for each enantiomer, with correlation coefficients larger than 0.99 for both enantiomers. The method was applied successfully in a preliminary study of urinary excretion after administration of CAR racemate to a healthy volunteer.

1. Introduction

Hypertension is one of the main factors that cause cardiovascular diseases and affect a large part of the world's population. According to Kearney et al., in 2000, there were 972.0 million hypertensive people and there is an estimate that, in 2025, this number will reach more than 1.5 billion people suffering from high blood pressure [1]. Many drugs for the treatment of hypertension, β-blockers, belonging to the class of adrenoceptors, have been widely used. The adrenoceptors are receptors directly related to the catecholamines (adrenaline and noradrenaline), being divided into α (alpha) and β (beta) classes. β-blockers are effective antihypertensive agents and, together with diuretics, have been the cornerstone of pioneering studies showing their benefits on cardiovascular morbidity and mortality because of blood pressure reduction in patients with hypertension [2].

Carvedilol (CAR) belongs to the third generation of β-blockers, which include, in addition to the β-blocking function, the vasodilator function due to the α-adrenoceptor [3,4]. CAR is a nonselective β-blocker that decreases the heart rate, and an α-blocker, which promotes the reduction in peripheral vascular resistance [5]. This drug is also a powerful antioxidant and neutralizer of oxygen radicals, as well as exerting other beneficial effects, such as inhibition of apoptosis [6] and improvement of myocardial postinfarction. In recent studies, CAR has shown preventive effects against skin cancer [7].

Although both enantiomers exhibit equally potent α-blocking activity and are administered as a racemic mixture of (+)-(*R*)- and (−)-(*S*)-CAR (Fig. S1), only the (−)-(*S*) enantiomer exerts β-blocking activity [8]. The pharmacokinetics study reports that CAR is rapidly absorbed after oral administration, with a half-life for elimination of about 8 h. Thus, the drug requires twice-daily dosing [9]. In healthy

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patients, less than 2% CAR can be found in urine [5,9,10]. In addition, CAR and other β-blockers are prohibited by the World Anti-Doping Agency in some sports. Therefore, considering the possible indications of CAR in several medical areas and because it is considered a doping agent, it is very important to develop simple, efficient and rapid methods for the determination of CAR in urine.

Methods for the enantioselective analysis of CAR in biological fluids include the use of chiral stationary-phase columns (direct methods) or derivatization (indirect methods). In direct methods, the column has a chiral selector, which is connected to the stationary phase, being responsible for the separation of enantiomers. This kind of method has been reported in the literature using different columns: Teicoplanin® column (normal phase) [11] and Chirobiotic® T (polar organic mode) [12] based on teicoplanin antibiotic, Chiralpak® AD (normal phase) based on amylose tris(3,5-dimethylphenylcarbamate) [13], Chiralcel® OD-RH (reverse phase) on cellulose tris(3,5-dimethylphenylcarbamate) [14,15], Sino-Chiral® AD (reverse phase) based on amylose tris(3,5-dimethylphenylcarbamate) [16] and Chirobiotic® V column based on vancomycin antibiotic [17]. These columns were coupled to a high-performance liquid chromatography system with fluorescence detection (HPLC–FL) [11,13–15] or mass spectrometry (LC–MS–MS) [12,15–17]. Indirect methods added an enantiomerically pure chiral reagent to the drug solution resulting in the formation of diastereoisomeric derivatives. Studies for chiral separation of CAR enantiomers employed 2,3,4,6-tetra-*O*-acetyl-β-*d*-glucopyranosyl isothiocyanate or menthyl chloroformate [18–22] and analyzed using LC–MS–MS [18,19,22] or HPLC–FL [20,21].

An efficient and simple method of sample preparation is required to extract CAR from a biological fluid. There are many techniques; the most used are those that employ adsorbent materials, however, when working with complex matrices, which have high amount of interference, employing materials with high selectivity makes the extraction easier. Molecularly imprinted polymers (MIP) revolutionized the chemistry of materials for sample preparation, which can mimic the behavior of sorption sites of natural receptors, i.e., the ability to recognize and bind to the desired molecular target, which is called a "template." Besides selectivity toward the target molecules or/and analogues, MIP possesses the characteristics of chemical and thermal stability, possibility to be used in a wide pH range, resistance to high temperatures, good adsorption capacity and reusability [23–27]. MIP components include template molecule, functional monomer, cross-linker, radical initiator and solvent, which should solubilize the other reagents, without reacting with each other [26,27].

Theoretical studies employing density functional theory (DFT) have been developed for evaluating the interaction between the template and functional monomer [28]. Only some papers reported in the literature included the solvent effect in their calculations. The template interacts by covalent or intermolecular interactions with monomer [29]. Subsequently, a cross-linker is added to promote the polymer synthesis, to form a rigid polymeric matrix [27] that is highly cross-linked, generating a three-dimensional polymer [23]. The polymerization reaction is initiated with the addition of an initiator. Finally, the template is removed from the polymeric matrix using a solvent or, if the template establishes a covalent bond with the monomer, a chemical cleavage is necessary [26,27]. The polymer presents similar cavities in size, shape and features to the template [24].

A pipette tip based on solid-phase extraction (PT–SPE) is one of the most promising and simplest techniques for miniaturized SPE. It differs from common SPE because only a small amount of sorbent is necessary to perform the extraction inside a polypropylene volumetric pipette tip (1000 μL), which serves as the extraction holder [30]. MIPs as sorbents in PT–SPE are good alternatives for these biological samples because they are capable of exhibiting specificity for a target analyte or a structurally related class of compounds [30–36]. Miniaturized SPE comprises four steps for the extraction process. First, a solvent, usually water, is passed to activate and accommodate the material, so that the sample and other solvents come in contact with the material uniformly. In the second step, the sample is passed, when retention of the analyte and interfering occurs. The third step consists in washing the material to remove interfering compounds from the sample. Finally, in the last step, a solvent is percolated for removal of the analyte. After elution, the eluent is evaporated and the residue resuspended in a solvent that is compatible with the analytical instrument [37].

We were able to join in the same work a rational synthesis through DFT calculations of a specific material based on MIP capable of extracting CAR from biological fluids using a miniaturized technique, i.e., we developed a simple, rapid, economical and miniaturized PT–MIP–SPE coupled to HPLC–DAD (diode array detector) to determine CAR enantiomers in human urine samples. All parameters that influence the recovery were systematically studied and discussed in detail. Finally, the validated method was applied in a preliminary study of urinary excretion (7 h) after administration of 12.5 mg CAR to a healthy volunteer.

2. Experimental

2.1. Materials and reagents

Reagents used in the synthesis of the MIP, i.e., methacrylic acid (MAA), ethylene glycol dimethacrylate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 4,4′-azobis(4-cyanovaleric acid) from Santa Cruz Biotechnology (Dallas, TX, USA). Carvedilol (CAR, 98%) was obtained from Fluka (Laramie, WY, USA). HPLC grade solvents used in the synthesis (chloroform), mobile phase (methanol and ethanol) and extraction (acetonitrile) were obtained from J.T. Baker (Mexico City, MX, Mexico). Other reagents, namely acetic acid, diethylamine, sodium chloride, toluene, hexane and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA), Synth (Diadema, SP, Brazil), Qhemis (Indaiatuba, SP, Brazil), Vetec (Rio de Janeiro, RJ, Brazil), Vetec (Rio de Janeiro, RJ, Brazil) and Macron Chemicals (Phillipsburg, NJ, USA), respectively. Water was distilled and purified using a Millipore Milli-Q Plus system (Bedford, MA, USA).

2.2. Preparation of standard solutions

The standard solution was prepared by weighing on an analytical balance 25.51 mg of CAR (98%) being transferred to a 25 mL volumetric flask and completed with methanol to obtain a CAR solution with a concentration of 1000 μg mL^{-1}. A 10 mL aliquot of this solution was transferred to a volumetric flask (50 mL) and made up with methanol to obtain a solution with a concentration of 200.0 μg mL^{-1} for intial tests. The solution with a concentration of 1000 μg mL^{-1} was used in serial dilutions to obtain the following concentrations for the CAR standards: 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0 μg mL^{-1}. These solutions were stored at −20 °C and protected from light in an amber flask.

2.3. Instrument

The separation of CAR enantiomers was performed using HPLC–DAD, Agilent® (Palo Alto, CA, USA) consisting of a model 1260 quaternary pump (G1311B), a model 1290 thermostat (G1330B), an autosampler model 1260 Hip ALS (G1367E), a column oven model 1290 TCC (G1316C) and a DAD model 1260 VL+ (G1315C). Data were acquired and controlled by the Agilent Open LAB Chromatography Data System® software. Separations were performed in reverse mode, using Chiralpak® IA column (100 mm × 4.6 mm, 3 μm) coupled precolumn C12 Phenomenex® AJO-6074 (4.0 mm × 3.0 mm), mobile phase consisting of methanol, ethanol and by mixing water with diethylamine (0.3%) in the proportions 64:15:21 (v/v/v) at a flow rate of 1.5 mL min^{-1}, temperature of 35 °C and 30 μL of injected volume. The chromatographic data were obtained at 240.0 nm.