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Online extraction of antihypertensive drugs and their metabolites from untreated human serum samples using restricted access carbon nanotubes in a column switching liquid chromatography system

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ABSTRACT

A novel analytical method was developed to determine 5 antihypertensive drugs of different pharmacological classes (angiotensin-converting enzyme inhibitors, calcium channel blockers, α-2 adrenergic receptor agonists, angiotensin II receptor blockers, and aldosterone receptor antagonists) and some of their metabolites in human serum. The untreated samples were directly analyzed in a column switching system using an extraction column packed with restricted access carbon nanotubes (RACNTs) in an ultra-high performance liquid chromatography coupled to a mass spectrometer (UHPLC–MS/MS). The RACNTs column was able to exclude approximately 100% of proteins from the samples in 2.0 min, maintaining the same performance for about 300 analytical cycles. The method was validated in accordance with Food and Drug Administration (FDA) guidelines, being linear for all the determined analytes in their respective analytical ranges (coefficients of determination higher than 0.99) with limits of detection (LODs) and quantification (LOQs) ranging from 0.09 to 10.85 $\mu g\,L^{-1}$ and from 0.30 to 36.17 $\mu g\,L^{-1}$, respectively. High recovery values (88–112%) were obtained as well as suitable results for inter and intra-assay accuracy and precision. The method provided an analytical frequency of 5 samples per hour, including the sample preparation and separation/detection steps. The validated method was successfully used to analyze human serum samples of patients undergoing treatment with antihypertensive drugs, being useful for pharmacometabolomic, pharmacogenomic, and pharmacokinetic studies.

1. Introduction

Arterial hypertension (AH) is an important and common risk factor for cardiovascular diseases (CVD) [1]. The undiagnosed and uncontrolled hypertension remains a public health challenge, affecting approximately one in four adults around the world, resulting in over ten million deaths annually [2,3]. In Brazil, this disease affects an average of 31 million people, and only about 23% of these patients have appropriately controlled blood pressure [4,5]. One of the factors that influences this low rate is the resistant hypertension (RH), which occurs when the blood pressure cannot be controlled despite the treatment with 1 diuretic and 2 or more antihypertensive drugs with different mechanisms of action, as well as in adequate doses [6]. It is estimated that around 10–15% of patients with AH are considered resistant with considerable risk of cardiovascular morbidity and mortality [7–9].

Therefore, methods to simultaneously analyze antihypertensive drugs can be important in studies of these critically ill patients. There are different analytical methods to determine drugs in biological matrices such as blood, urine and tissues [10–13]. This drug monitoring assists in the therapy optimization, particularly when the used drug has a narrow therapeutic range, besides providing the understanding of serum drug concentrations and, consequently, propitiating an effective therapy with minimized adverse effects [14,15].

Usually, untreated biological samples cannot be directly analyzed by an analytical technique due to their complex composition. In this case, a sample preparation step is needed to clean samples

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and make them compatible with techniques and analytical equipment. An alternative to the classical sample preparation techniques has been the use of the restricted access materials (RAMs). These materials are intelligent sorbents that have outer surfaces able to exclude macromolecules (chemically or physically) and inner surfaces that retain the low-molecular weight compounds (inorganic or organic) [16–19]. They often have been used in column switching liquid chromatography analyses, allowing that raw complex biological samples can be directly injected in the online system, minimizing sample handling and preparation time [19]. Several conventional sorbents have been converted to RAMs by chemical modifications (e.g., silica [20,21], polymers [22,23], among others), resulting in materials with the advantageous characteristics of their precursors, in addition to the ability of excluding macromolecules, provided by the chemical modifications.

Online sample preparation provides many good features, making the analytical methods faster, easier, with higher reproducibility, and cheaper. However, some difficulties related to the matrix components may occur when this type of configuration is used to determine compounds in complex biological matrices directly injected into the system [24]. Proteins, saccharides and lipids can interfere in the analyses, principally due to the propensity of these substances to be retained by the sorbent and the high probability of these trapped compounds to interfere in the analytical response. The serum is a matrix with a high percentage of lipids, and its analysis can be quite challenging [24]. Therefore, it is necessary a detailed study, mainly of the matrix effect, to ensure that the methodology is free from such interferences, as demonstrated by Schug and colleagues in their studies [24–26].

Currently, carbon nanotubes (CNTs) have been commonly used in solid phase extraction (SPE) due to their high ability to preconcentrate both organic [27–29] and inorganic compounds [30–32]. This material has unique tubular structures that give it excellent properties, such as an extremely large surface area and thermal stability. They are classified as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), being the number of walls proportional to their adsorption capacity for chemicals. Despite the successful use of the CNTs as sorbent in sample preparation, this material has some limitations when used in raw biological sample treatments. In fact, the proteins (present in these samples in high concentrations) can be retained on CNT surfaces, resulting in a decrease in the adsorption capacity, as well as in a blockage of the columns packed with this sorbent. A feasible solution can be the conversion of MWCNTs in RAMs.

In 2015, our research group synthesized the first RAM based on CNTs, which was called restricted access carbon nanotubes (RACNTs) [33]. This material was obtained by modifying the CNTs with a bovine serum albumin (BSA) layer, chemically crosslinked. Thus, when complex biological samples are percolated through a column packed with RACNTs, low molecular weight compounds penetrate into the pores of the BSA layer, interacting with the core of CNTs. At the same time, the proteins from the sample are excluded due to an electrostatic repulsion between them and those on the BSA coating [33–35]. This electrostatic repulsion occurs when the sample pH is lower or higher than the isoelectric point (IP) of proteins (both from the sample and from the RACNTs layer), which assume positive or negative charges, respectively [36].

RACNTs were initially applied to directly extract Cd^{2+} [33] and Pb^{2+} [35] from raw human blood serum samples in an on-line SPE system with detection by thermospray flame furnace atomic absorption spectrometry (TS-FF-AAS). The material was efficient to retain the ions and exclude macromolecules at least for 200 sequential extractions, with precision and accuracy. Recently, 2 methods using RACNTs in a column switching liquid chromatography system were developed to determine organic compounds (tetracyclines in cow milk samples [34] and anticonvulsants in human plasma samples [37]). Both methods had good figures of merit and each column was used for at least 250 cycles with the same performance. Additionally, these were the first applications of RACNTs to extract organic compounds from complex biological matrices.

To the best of our knowledge, RACNTs were previously used only for 4 applications [33–35,37]. Moreover, in the methods developed for the determination of organic compounds, only few molecules of the same class (4 tetracyclines [15] and 3 anticonvulsants [18]) were determined, despite the high capacity of the CNTs of adsorbing different compounds at the same time. The main hypothesis of this paper is that RACNTs can also be simultaneously used to extract compounds of different classes and with different structures and polarities. To test this hypothesis, we developed a column switching liquid chromatography method using a RACNTs column to determine 5 structurally different antihypertensive drugs: enalapril (ELP); losartan (LOS); amlodipine (AML); clonidine (CND); spironolactone (SPL); and some of their main metabolites, enalaprilate (ENT); losartan carboxylic acid (LCA); and losartan N2-glucuronide (N2G), in human serum.

2. Experimental

2.1. Liquid chromatography and mass spectrometry conditions

The antihypertensive drugs analyses were carried out using an ultra-high performance liquid chromatography system (UHPLC) model LC–MS 8030 equipped with a triple-quadrupole mass analyzer (Shimadzu®, Kyoto, Japan). The system was equipped with a UV detector model SPD-10AVP, 3 LC-20AD pumps, an auto sampler SIL-20 AHT (all obtained from Shimadzu®), an electronic 6-port switching valve, model 11R-0016H (Valco®, Houston, TX, USA), and an analytical column Shim-pack XR-ODS C18 (150 mm × 4.6 mm, 5 μm; L x ID, particle size) (from Shimadzu®). Treatment and data acquisition were performed using LabSolutions® software. To obtain the extraction column, an empty guard-column Shimpack G-ODS® (1 cm x 4 mm, L x ID) containing 2 column frits on each side with a pore size of 0.5 μm was packed with approximately 30 mg of RACNTs. The positive electrospray ionization mode (ESI +) was chosen, and the optimal collision energies as well as the multiple reaction monitoring (MRM) transitions were optimized separately for each analyte (the optimized conditions can be seen in Table 1). The identification of each analyte was obtained from the presence of 3 fragments, and the quantitation was performed using the most abundant originated fragment for each molecule (Table 1). The oven, source block, and desolvation temperatures were 70, 400, and 250 °C, respectively. The flow rates adopted were 2.0 and 15.0 mL min^{-1} to the nebulizing and drying gas, respectively. The sample volume injected into the system was 100 μL.

2.2. Reagents and solutions

The analytical standards enalapril (purity ≥ 98%), enalaprilat (purity ≥ 99%), (s)-amlodipine (purity ≥ 99%), losartan (purity ≥ 95%), losartan carboxylic acid (purity of 100%), and losartan N2-glucuronide (purity of 100%) were purchased from Santa Cruz Biotechnology® (Dallas, TX, USA), clonidine hydrochloride (purity ≥ 99%) from Tocris Bioscience® (Bristol, United Kingdom), and spironolactone (purity ≥ 99%) from Acros Organics® (Geel, Belgium). The internal standards (ISs) losartan-d4 (LOS-d4) (purity of 100%) and clonidine-d4 hydrochloride (CND-d4) (purity of 100%) were purchased from TRC® (Toronto, Canada). Ultra-high purity water (182 Ω.m) from a Milli-Q system (Millipore®, Bedford, MA, USA) was used in the preparation of solutions. Stock solutions at a concentration of 1 g L^{-1} to each molecule were prepared by dissolving each compound in methanol. After preparation, these

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