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Rapid and direct analysis of statins in human plasma by column-switching liquid chromatography with restricted-access material

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ABSTRACT

This study presents the development of a column-switching liquid chromatographic method with direct injection of human plasma for simultaneous determination of four statins (lovastatin, pravastatin, rosuvastatin and simvastatin), the main class of drugs used in the treatment of hyperlipidemia. By using a C18 (30 mm × 4.6 mm, 15 μm) a lab made bovine serum albumin restricted access material (RAM) column was prepared and compared with a commercial alquil-diol silica RAM column (C18, 25 mm × 4.0 mm, 25 μm) in terms of their protein exclusion capacity and micromolecules retention. Foreflush and backflush modes were compared for both RAM columns to the number of theoretical plates, asymmetry, resolution and chromatographic run time. The developed method was validated in the range from 125 to 876 ng mL^{-1} for lovastatin, rosuvastatin and simvastatin, and from 500 to 2000 ng mL^{-1} for pravastatin, presenting selectivity, precision and accuracy intra and inter-run. Total analysis time (sample preparation and chromatographic separation) was only 16 min when the backflush mode was employed in the column-switching system.

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1. Introduction

Hyperlipidemia, characterized by high plasma levels of triglycerides or cholesterol, results from a disturbance in the synthesis and degradation of lipoproteins. Raised cholesterol is the major risk factor for atherosclerosis and cardiovascular disease, including coronary heart disease [1]. Overall, raised cholesterol is estimated to cause 2.6 million deaths (4.5% of total) and 29.7 million disability adjusted life years [2]. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, also known as statins, are the main pharmacological group employed in the treatment of hyperlipidemia. Statins inhibit the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis, leading to a reduction in the plasma low density lipoprotein (LDL)-cholesterol level [3].

The analysis of statins in plasma samples is essential in pharmacokinetic and bioequivalence studies and therapeutic drug monitoring. Generally, drug analysis in complex matrices involves four steps: sample preparation, analytical separation, detection and data handling [4]. High performance liquid chromatography (HPLC), a well-established and highly efficient tool, is widely used as separation technique in drug analysis. On the other hand, sample preparation still needs more improvement, since conventional procedures such as protein precipitation, liquid–liquid extraction, and solid phase extraction in the off-line mode have been extensively used. These techniques are time and solvent consuming and expose the analyst to contact with potentially contaminated biological samples [5]. Automated column-switching is an attractive approach to be used as alternative to the traditional sample preparation techniques. In a multidimensional system, restricted access material (RAM) column performs exclusion/extraction in the first dimension and an analytical column separates the analytes in the second dimension. Using this approach the direct injection of plasma samples in the chromatograph is allowed [6].

Most of the methods described in the literature dealing with statins analysis employed conventional methods for sample preparation. Burugula et al. presented a method to determine simvastatin and sitagliptin in human plasma. A tedious and time consuming liquid–liquid extraction method was used [7]. A protein precipitation method followed by liquid–liquid extraction was developed

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for the simultaneous quantification of simvastatin and simvastatin acid in human plasma [8]. Solid phase extraction was also used for simvastatin analysis [9] as well as column-switching approaches. However, extensive additional procedures were needed to extract the analytes [10,11]. Different methods for lovastatin and pravastatin determination have been described in the literature. The majority of them employed liquid–liquid and solid phase extraction for lovastatin [12–14] and pravastatin [15–17], respectively. Methods for rosuvastatin analysis in human plasma employing protein precipitation [18], liquid–liquid extraction [19] and solid phase extraction [20] have also been found.

In this context, the purpose of this study was to develop a fully automated liquid chromatography column-switching system for simultaneous analysis of lovastatin (LOV), pravastatin (PRA), rosuvastatin (ROS) and simvastatin (SIM) (Fig. 1) in human plasma samples. From our knowledge, it is the first time that an automated method with direct injection of plasma samples was described for simultaneous determination of these four statins. A lab made bovine serum albumin RAM column (RAM-BSA) was prepared and compared with a commercial alquil-diol silica RAM column (RAM-ADS). The method was optimized and fully validated according to current Brazilian legislation.

2. Experimental

2.1. Analytical standards, reagents and materials

Calcium atorvastatin (ATO) (Fig. 1), employed as internal standard, sodium pravastatin, simvastatin, ketoprofen, sodium diclofenac, omeprazole, teofilin, mebendazole, propranolol and trimethoprim analytical standards were provided by United States Pharmacopeia (Rockville, MD, USA). Calcium rosuvastatin, lovastatin and bromazepam were from PGS Purity Grade Standard (Carrboro, NC, USA), Pharmanostra (Rio de Janeiro, Brasil) and European Pharmacopeia (Strasbourg, France), respectively. Acetonitrile and methanol HPLC grade were from J. T. Baker (Center Valley, PA, USA). Formic acid, Labsynth (Diadema, Brazil), orthophosphoric acid and sodium borohydride, Vetec (Duque de Caxias, Brazil), anhydrous ethyl alcohol, Coomassie brilliant blue G-250, dibasic potassium phosphate, monobasic potassium phosphate, glutaraldehyde 25% (v/v) and potassium hydroxide, Sigma–Aldrich (Steinheim, Germany) were all analytical grade. Bovine serum albumin (BSA) fraction V (98% of purity) was from Inlab (São Paulo, Brazil). Water was purified using Millipore Direct Q3 Milli-Q equipment (Bedford, MA, USA). Drug-free plasma was collected at Hematology Laboratory from Faculdade de Farmácia da Universidade Federal de Minas Gerais (UFMG) and maintained frozen at −70 °C. This study was approved by UFMG Ethical Committee.

An ACE (Aberdeen, Scotland) C18 (30 mm × 4.6 mm, 15 μm) column was modified in situ with BSA in order to obtain the RAM support. A Lichrocart 25-4 Lichrospher C18 ADS (RAM-ADS – 25 mm × 4.0 mm, 25 μm) and a Lichrocart 125-4 Purospher Star C8 (125 mm × 4.0 mm, 5 μm) analytical column were from Merck (Darmstadt, Germany).

2.2. Column-switching system

The column-switching system was assembled in a Shimadzu (Kyoto, Japan) liquid chromatograph equipped with a SCL-10A VP controller, two LC-10AD VP pumps, two FCV-10 AL VP quaternary solenoid valve block, a CTO-10A VP oven, a SIL-10AD VP autoinjector, and SPD-M10A VP diode array detector. The pumps, as well as autoinjector and detector, were properly coupled to a six-port and two positions valve from Valco (Houston, TX, USA). Class-VP software (Shimadzu) controlled all the events of the system, as well as the switching valve microelectric actuator. The column-switching system was used in the backflush and foreflush modes (Fig. 2).

In the backflush mode, plasma sample was directly injected in the RAM column and was eluted by the aqueous mobile phase from pump A (position A). Simultaneously, the analytical column was conditioned by the mobile phase from pump B. With the switching valve set in position A, injection of the sample was performed and macromolecules were excluded through the RAM column. Then, the mobile phase from pump B was directed to RAM column on the opposite direction of the sample injection (position B), carrying the analytes to the analytical column and detector. In the foreflush mode, plasma sample was also directly injected in the RAM column and was eluted by the aqueous mobile phase from pump A (position A). After injection and macromolecules exclusion, the valve was switched to position B. At position B the mobile phase from pump A was changed to separation mobile phase and directed to RAM column in the same direction of the sample injection. Then, the analytes were eluted to analytical column and detector.

2.3. BSA column preparation and evaluation

RAM-BSA column was in situ prepared in an ACE C18 (30 mm × 4.6 mm, 15 μm) column by adapting a protocol described elsewhere [21]. Briefly, the procedure was as follows: with the aid of an infusion pump at 1.0 mL min^{-1}, the support was subsequently eluted with 0.05 M (pH 6.0) potassium phosphate buffer for 20 min, bovine serum albumin at 1.0 mg mL^{-1}, prepared in the same buffer, for 30 min, deionized water for 2 min, and 25% (v/v) glutaraldehyde for 15 min. After 6 h, the column was flushed with a 1.0 mg mL^{-1} aqueous solution of sodium until pH 10 has been achieved. After 2 h, the support was washed with deionized water for 1 h. The RAM-BSA column was stored in water at 2 °C after preparation and use.

The exclusion capability of RAM-BSA homemade and RAM-ADS were evaluated and compared, by means of Bradford protein assay [22]. By using the chromatographic system on the unidimensional mode, the excluded proteins by the RAM columns were evaluated. RAM-BSA column was conditioned with water at 1.0 mL min^{-1} for 10 min. Then, 50, 100, 200 and 500 μL of plasma samples were injected (in triplicate) and eluted with the same solvent and flow-rate. Two fractions of the eluate were collected, at minutes 2 and 4 of the chromatographic run. The same procedure was performed with RAM-ADS. Plasma samples used as 100% proteins reference were prepared by diluting 50, 100, 200 and 500 μL of plasma in water to a final volume of 2 mL. Five milliliters of the Bradford reagent (Coomassie brilliant blue G-250 at 100 mg mL^{-1}) were added to 500 μL of the eluate from the RAM columns and allowed to stand for 3 min. Spectrophotometric measures were performed at 595 nm, using a mixture of 500 μL of water and 5.0 mL of the Bradford reagent as blank. The same procedure was accomplished with the reference plasma samples. Scanning of the two fractions (2 and 4 min) and of the Bradford reagent between 190 and 800 nm were also performed. Finally, the exclusion capability of RAM columns was calculated, comparing the absorbances of the collected and the reference samples. The exclusion capabilities of RAM-BSA and RAM-ADS were statistically compared by means of Student's *t*-test.

Nine drugs with acid (ketoprofen, diclofenac and pravastatin), neutral (bromazepam, omeprazole and teofilin) and basic (mebendazole, propranolol and trimethoprim) properties were used to evaluate the retention factor (k) and asymmetry (As) with RAM-BSA and RAM-ADS columns. Mobile phase composed by acetonitrile and phosphate solution 0.1 M at pH 2.5; 6.0; 6.5 and 7.0 (40:60) was employed. Twenty microliters of these drugs prepared in the mobile phases at 20 μg mL^{-1} were injected on the unidimensional mode. The detector was set at the maximum absorbance of each drug and temperature was 35 °C. Acetonitrile was used as void volume marker.

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