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Determination of total and unbound concentrations of lopinavir in plasma using liquid chromatography–tandem mass spectrometry and ultrafiltration methods

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ABSTRACT

Lopinavir is an HIV protease inhibitor with high protein binding (98–99%) in human plasma. This study was designed to develop an ultrafiltration method to measure the unbound concentrations of lopinavir overcoming the non-specific binding issue. A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of total concentrations of lopinavir in plasma was developed and validated, and an adaptation was also optimized and validated for the determination of unbound concentrations. The chromatographic separation was performed with a C_{18} column (100 mm × 2.1 mm i.d., 5 μm particle size) using a mobile phase containing deionized water with formic acid, and acetonitrile, with gradient elution at a flow-rate of 350 μL min^{-1}. Identification of the compounds was performed by multiple reaction monitoring, using electrospray ionization in positive ion mode. The method was validated over a clinical range of 0.01–1 μg/mL for human plasma ultrafiltrate and 0.1–15 μg/mL in human plasma. The inter and intra-assay accuracies and precisions were between 0.23% and 11.37% for total lopinavir concentrations, and between 3.50% and 13.30% for plasma ultrafiltrate (unbound concentration). The ultrafiltration method described allows an accurate separation of the unbound fraction of lopinavir, circumscribing the loss of drug by nonspecific binding (NSB), and the validated LC–MS/MS methodology proposed is suitable for the determination of total and unbound concentrations of lopinavir in clinical practice.

1. Introduction

Lopinavir (LPV) is an HIV protease inhibitor (PI) drug used for the treatment of human immunodeficiency virus (HIV) infection. As most of the PIs, it is a large, lipophilic and weakly basic drug, highly bound to plasma proteins (98–99%), mainly to α_1-acid glycoprotein (AAG) [1]. Since the unbound fraction of the drugs in plasma is usually considered the only that can exert its activity, the bound of a drug to protein limits its pharmacological actions. Therapeutic drug monitoring (TDM) based on total plasma concentrations is currently performed for the antiretroviral drugs (ARVs) [2,3]. However, different parameters can significantly change unbound fractions, having an influence on the clearance and distribution of some PIs; that is the high interindividual differences in protein binding, and also the phenotypic variants of AAG recently identified [4]. In this way, differences on unbound fractions are also observed in some physiological or pathological conditions, such as pregnancy or infections [5,6]. Therefore, the quantification of LPV unbound concentrations can be useful in some specific populations and/or conditions.

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Several methodologies by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) have been published for the simultaneous determination of total concentrations of LPV in plasma, either with other PIs [7–14], or with PIs and non-nucleoside reverse transcriptase inhibitors (NNRTI) [15–24]. Several available data exist for the quantification of LPV in ultrafiltrate [25–28,11,29,30]; however, its methodology has scarcely been full validated. The two most used methods to determinate plasma unbound concentrations are ultrafiltration (UF) and equilibrium dialysis (ED) [31]. Although UF increases the analysis throughout compared with ED, as it is less time consuming, it has also been associated to the non-specific binding (NSB) problem, especially present for higher lipophilic drugs, observed with ED [32]. Several approaches of pre-treatment of the filter and some modifications of the UF methodology have been applied in order to reduce the NSB of drugs to the filter membranes or glass and plastic recipients [33,34], but they cannot always eliminate sufficiently the NSB. In this way, a different NSB behavior of a compound between its incubation with an ultrafiltration device in PBS versus plasma has been recently reported, which could explain these differences.

The aim of this study was to develop and validate an LC–MS/MS method to determine lopinavir plasma concentrations, and to adapt it to accurately measure unbound concentrations in plasma, after separation of the protein-bound component; several approaches were conducted for developing an ultrafiltration procedure that overcomes the NSB limitation associated to UF. This work presents the development and validation of an ultrafiltration method for the separation of unbound fractions of LPV, as well as of the LC–MS/MS methodologies used for the determination of lopinavir total and unbound concentrations of LPV in plasma.

2. Experimental

2.1. Chemicals and reagents

Lopinavir was procured from Abbot Laboratories (Suresnes, France) and deuterated lopinavir (Lopinavir-d8) by Toronto Research Chemicals (North York, ON, Canada). Formic acid (FA), methanol (MeOH), acetonitrile (ACN) and water, all in LC/MS grade, were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Tween-20 used was also from Sigma–Aldrich. Amicon Centrifree® Filter System (molecular weight cutoff 30 kDa) was from Millipore Corporation. Drug free plasma was purchased from Utak Laboratories (Valencia, Spain) and stored at −20 °C.

2.2. Equipment

LC analyses were carried out on an Accela® System liquid chromatograph (Fisher Scientific, Les Ulis, France) using a Kinetex C_{18} column (100 mm × 2.1 mm i.d., 5 μm particle size) (Phenomenex, Le Pecq Cedex, France) protected with an inline filter. The column temperature was set to 30 °C. Chromatographic separation was achieved by gradient elution at a flow rate of 350 μL/min. Mobile phases used were deionized water with 0.1% formic acid (A) and acetonitrile (B). At time zero the flow consisted of 60% of mobile phase A, which was decreased linearly to 5% over 5 min. After 1.5 min, the composition of the mobile phase was set to initial conditions (60% A and 40% B) in 0.1 min, and the column was equilibrated for 2.4 min prior the next injection. The total run time including column equilibration was 9 min.

The liquid chromatograph was coupled to a Thermo-Finnigan TSQ Quantum Discovery Max triple quadrupole mass-spectrometer through Heated-ElectroSpray Ionisation (H-ESI) interface in positive mode in the multiple reaction monitoring (MRM) mode. The following optimized conditions were used: capillary voltage, 5 kV; temperature of the gas transfer, 300 °C; sheath gas, 45 psi; auxiliary gas, 25 psi. Nitrogen was obtained by a NM 30 LA generator (Lab Gaz System, Massy, France) and was employed as nebulizing and auxiliary gas. In order to establish the MRM transitions and compound specific parameters, standard solutions of the compounds (10 μg/mL) were infused into the MS at 5 μL min^{-1} with mobile phase (50:50, A:B) at 200 μL/min.

Instrument control and data collection were handled by computer equipped with Xcalibur software (Version 2.0, Fisher Scientific).

2.3. Preparation of calibration standards and quality control samples

Stock solutions of lopinavir (1 mg/mL) were prepared by dissolving 10 mg of the analyte in 10 mL of methanol, one for calibration standards (CAL) and one for validation samples (VS). A stock solution of the internal standard was also prepared in methanol with an approximate concentration of 1 mg/mL. Working solutions of the internal standard of 1 and 0.1 μg/mL were prepared by 1:1000 and 1:10,000 dilution of the 1 mg/mL stock solution in methanol. For the preparation of calibration standards and validation samples, working solutions of 100, 10, 1 and 0.1 μg/mL were prepared by 1:10, 1:100, 1:1000 and 1:10,000 dilutions of the respective 1 mg/mL stock solution of lopinavir with methanol. All solutions were stored at −20 °C until use.

Calibration standards in the range of 0.1–15 μg/mL for the total concentration and of 0.01–1 μg/mL for the unbound concentration were prepared by addition of appropriate amounts of working solutions to blank plasma and blank ultrafiltrate, respectively.

To obtain validation samples of 0.1, 0.3, 4 and 12 μg/mL for the total concentration and of 0.01, 0.03, 0.4 and 0.7 μg/mL for the unbound concentration, appropriate amounts of working solutions were added to blank plasma and blank ultrafiltrate, respectively.

2.4. Sample preparation

For the analysis of total concentrations of LPV, 50 μL of plasma was spiked with 5 μL of IS working solution of 1 μg/mL prior to protein precipitation with ACN (1 mL). Samples were vortexed and left to stand at room temperature for 15 min. After vortex mixing, samples were centrifuged at 12,000 rpm for 10 min. The supernatant (0.1 mL) was separated and 0.2 mL of 0.1% formic acid in water solution was added. Samples were then re-vortexed and centrifuged once again (12,000 rpm for 5 min) before transfer into a vial and LC–MS/MS analysis (5 μL injected).

For the analysis of the unbound concentrations, a dilution of calibration standards, validation samples and ultrafiltrate (obtained from samples) with an appropriate volume of methanol was done before its injection (5 μL).

2.5. Validation protocol

Analytical method validation for plasma and ultrafiltrate was performed according to the FDA and EMA guidelines for validation of bioanalytical assays [35,36].

The selectivity was verified in six different batches of control drug-free plasma by the absence of interfering compounds. The presence of carryover was evaluated by injecting two extracted blank matrix samples sequentially after the injection of an upper limit of quantification sample.

The extraction recovery in plasma, at three concentration levels (0.3, 4 and 12 μg/mL), was calculated by comparing the peak area ratios of the analyte to the internal standard obtained from the extracted spiked samples and the mean of the peak area ratios obtained when the analyte was added to extracted blank plasma