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Short communication

Determination of atazanavir in human plasma using solid-phase extraction and high-performance liquid chromatography

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Abstract

Atazanavir is a new HIV-1 protease inhibitor. A simple high-performance liquid chromatographic method using UV detection was developed and validated for the analysis of atazanavir in human plasma. The sample clean up was carried out using solid-phase extraction with OASIS® MCX cartridge. The chromatographic separation was achieved on a Kromasil® C_{18} (150 mm × 3 mm, 5 μm) column with a mobile phase consisting of acetonitrile and water (38:62 v/v) delivered isocratically. The effluent of the column was monitored at a wavelength of 210 nm. The assay was linear over the concentration range of 0.156 to 10 μg/ml and the limit of quantification was 0.156 μg/ml. The method was also validated with respect to recovery, precision, accuracy and specificity.

This method is suitable for therapeutic drug monitoring of atazanavir and can be easily reproduced with standard equipment.

Keywords: Reversed-phase chromatography; Solid-phase extraction; Protease inhibitors; Atazanavir; Therapeutic drug monitoring

1. Introduction

Atazanavir (ATZ) (Reyataz®) is a new azapeptide inhibitor of HIV-1 protease. ATZ possesses unique HIV resistance profile and favourable pharmacokinetic properties allowing once-daily dosing. Unlike other protease inhibitors (PIs), ATZ does not negatively impact the lipid profile.

Reyataz® was approved for treatment of HIV by the European Medicines Agency (EMEA) in 2004 and indicated in combination with other anti-HIV drugs. ATZ use is recommended boosted, with low dose ritonavir (rtv), which acts as a pharmacokinetic enhancer.

PIs are appropriate candidates for therapeutic drug monitoring (TDM) [1]: these compounds exhibit significant inter-individual pharmacokinetic variability and relationships between plasma concentrations and antiviral effect. In particular, sub-optimal concentrations of PIs limit drug efficacy. TDM is also of clinical relevance to prevent the appearance of toxic side effects.

As ATZ undergoes cytochrome P450 3A4 (CYP 3A4) metabolism, numerous drug–drug interactions are expected and TDM provides a useful tool to detect and manage them. Particularly, interactions may be very unpredictable using the new therapeutic option with double-boosted PIs [2], thus requiring TDM.

TDM also permits to document medication compliance as non-adherence to treatment affects the success of antiretroviral therapy and is related to virologic failure. As recommended by French guidelines for HIV treatment [3], TDM has been introduced in our centre as a part of multidisciplinary approach including physician, virologist and pharmacist [4]. This approach aims to optimize clinical use of antiretroviral drugs.

Only few analytical methods have been described for the determination of ATZ in biological samples. Most of

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them used liquid chromatography–tandem mass spectrometry (LC–MS–MS) [5–7]. LC–MS–MS systems are not available in all TDM routine laboratories and other analytical method need to be developed. Colombo et al. [8] have proposed a HPLC method using UV-diode array detection for the simultaneous analysis of PIs, including ATZ, and non-nucleoside reverse transcriptase inhibitors. However, this latter involves a 40 min gradient elution run, requiring equilibration step. Likewise, Dailly et al. [9] have recently proposed a method involving gradient elution and requiring multi-step, time-consuming liquid–liquid extraction.

In this paper, we report the development and validation of a simple solid-phase extraction followed by isocratic high-performance liquid chromatography (HPLC) assay with ultraviolet detection which allows rapid, precise, specific and sensitive quantification of ATZ in plasma.

2. Experimental

2.1. Reagents and solutions

Bristol-Myers Squibb (New Brunswick, NJ, USA) kindly supplied ATZ sulfate. The internal standard (IS), prazepam, was purchased from Sigma Chemical Company (Saint Quentin Fallavier, France). HPLC grade acetonitrile, methanol and 1 M HCl were purchased from Carlo Erba (Rueil Malmaison, France). Pure water was obtained from a Millipore Milli-Q plus water purification system (Saint Quentin en Yvelines, France). Acetic acid and ammonia solution were purchased from Merck (Strasbourg, France).

Drug free human plasma was purchased from centre atlantique blood transfusion centre (Nantes, France).

Solid-phase extraction cartridges (Oasis® MCX 1cc) were obtained from Waters (Saint Quentin en Yvelines, France).

Stock solutions of ATZ (400 μg/ml) and of IS (100 μg/ml) were both prepared in volumetric flasks by dissolving an accurately weighted amounts of ATZ sulfate or IS in methanol. All stock solutions were stored at +4 °C for 6 months.

ATZ working solutions were prepared from a 1/4 dilution of the stock solution in methanol.

The calibration plasma standards (0.156, 0.3125, 0.625, 1.25, 2.5, 5 and 10 μg/ml) were prepared using 1 ml of drug-free human plasma samples spiked with 100 μl standard solutions of ATZ (1.5625–100 μg/ml), obtained by appropriate dilution in methanol of the stock solution.

Plasma quality controls (QC) were also prepared using the same procedure in order to evaluate the precision, accuracy, recovery, quantification and detection limits of the method. These concentrations were based on human plasma levels usually determined in pharmacokinetic studies.

QC samples were prepared at three levels and stored at −20 °C until analysis:

- low level corresponding to the limit of quantification (LOQ): 0.156 μg/ml;
- middle level: 2 μg/ml;
- high level: 7 μg/ml.

2.2. HPLC system

The analytical column (Kromasil® C_{18}, 5 μm, 150 mm × 3 mm) and the ChromSep® C_{18} guard column (10 mm × 2 mm) were obtained from Varian (Les Ulis, France).

The HPLC system consisted of a L-6000 Merck pump (VWR, Fontenay-sous-Bois, France), a Waters 717 plus Autosampler injector (Saint Quentin en Yvelines, France), a Waters 2487 UV detector (Saint Quentin en Yvelines, France) and a PC with Kromasystem 2000 integrator from Bio-Tek instruments (Trappes, France).

The mobile phase consisted of a mixture of acetonitrile and water (38:62 v/v) and was filtered through a 0.2 μm nylon filter membrane (Lida Manufacturing Corp., USA) before use. The HPLC system was operated isocratically at a flow rate of 1.0 ml min^{-1} and the effluent of the column was monitored at a wavelength of 210 nm.

2.3. Plasma sample pre-treatment

Patient's blood samples were collected in tubes with anticoagulant (lithium heparinate) and centrifuged (3000 × *g* for 10 min at 20 °C). Plasma samples were then stored at −20 °C prior to analysis. They were handled according to the standard procedure for bio-hazardous samples.

3. Extraction technique

Fifty μl of IS stock solution and 100 μl of 5N acetic acid were added to calibration standard, quality control and patient plasma samples. After mixing on a Vortex® mixer for 30 s, plasma was transferred onto the solid-phase extraction cartridge conditioned with 1 ml methanol and 1 ml purified water. Then cartridge was washed with 1 ml NH_4OH–CH_3OH–H_2O (5:5:90 v/v/v) solution. Elution was finally carried out twice with 500 μl NH_4OH–CH_3OH (5:95 v/v) and the eluate was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and 50 μl were injected in the HPLC system.

3.1. Calibration and calculation procedures

Standard curves were constructed by plotting the peak height ratios of ATZ and IS versus ATZ concentrations. Then a linear regression was performed to calculate the ATZ concentrations of plasma samples from the peak height ratios.

3.2. Assay validation

Assay validation involves linearity, specificity, accuracy, precision, limit of detection (LOD) and limit of