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Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry

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Abstract

A sensitive and accurate liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the intracellular determination of nine antiretroviral drugs in human peripheral blood mononuclear cells (PBMCs) is proposed. PBMCs are isolated by density gradient centrifugation using Vacutainer™ CPT tubes and cell count is performed with a Coulter® instrument. Single-step extraction of drugs from PBMCs pellets is performed with MeOH 50% (with clozapine added as internal standard, I.S.) and the supernatant is injected onto a 2.1 mm × 30 mm SymmetryShield™ 3.5 μm-RP18 column equipped with a 2.1 × 10 mm guard column. Chromatographic separations are performed using a gradient program with a mixture of 2 mM ammonium acetate containing 0.1% formic acid and acetonitrile with 0.1% formic acid. Analytes quantification is performed by electro-spray ionisation–triple quadrupole mass spectrometry using the selected reaction monitoring (SRM) detection mode. The positive mode is used for the HIV protease inhibitors (PIs) indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine, and the negative mode is applied for efavirenz. The calibration curves are prepared using blank PBMCs spiked with antiretroviral drugs at concentrations ranging from 0.5 to 100 ng/ml of cell extracts and fitted to a quadratic regression model weighted by 1/(concentration)^2. The lower limit of quantification is less than 0.5 ng/ml. The mean extraction recovery for all PIs/NNRTIs is always above 88%. The method is precise, with mean inter-day CV% within 0.6–10.2%, and accurate (range of inter-day deviation from nominal values −7.2 to +8.3%). This analytical method can be conveniently used in clinical research for the assessment of intracellular levels of all PIs/NNRTIs commercially available at present using a simple one-step cell extraction of PBMCs followed by liquid chromatography coupled with tandem triple quadripole mass detection.

Keywords: Protease inhibitors; Non-nucleoside reverse transcriptase inhibitors; PBMCs; Peripheral blood mononuclear cells; HPLC–MS/MS; Indinavir; Amprenavir; Saquinavir; Ritonavir; Nelfinavir; Lopinavir; Atazanavir; Efavirenz; Nevirapine

1. Introduction

Major advances have been accomplished in recent years for the treatment of human immunodeficiency virus type 1 (HIV-1) infected patients. However, many patients experience treatment failure within one year after initiation of antiretroviral therapy [1]. Drug resistance due to mutations of

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the viral genome accounts for a large proportion of treatment failures [2–4]. The emergence of genetic changes in the genome of HIV-1 is fostered by ongoing viral replication in the presence of sub-inhibitory concentrations of antiretroviral drugs. Poor penetration of drugs into various profound compartments of the body (sanctuary sites), inadequate treatment adherence, and variability in drug pharmacokinetics may contribute to the occurrence of sub-therapeutic drug level in vivo.

Therapeutic drug monitoring (TDM) of HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) considers the adjustment of total plasma concentrations as a mean to optimise response to antiretroviral therapy [5]. However, only the fraction reaching the intracellular compartment is reasonably expected to exert an antiviral action [6,7]. Whereas PIs and NNRTIs (Figs. 1 and 2) are two therapeutic class of drugs showing distinct physicochemical properties, both are mostly lipophilic in nature and are assumed to enter cells by passive diffusion through the cellular phospholipidic bilayer membrane. However, the intimate mechanisms by which antiretroviral drugs accumulate within cells remains generally unknown. Previous in vitro [7] and in vivo [8] studies have shown striking differences in the intracellular kinetics of PIs. Similarly, a marked variability has been observed in their accumulation in lymphoblastoid cell lines in vitro, and in peripheral blood mononuclear cells (PBMCs) in vivo [9–11]. Moreover, a number of transmembrane transport proteins, such as P-glycoprotein, the gene product of *ABCB*1 (*MDR*1), and related ABC (ATP binding cassette) transporters are known to actively expel drugs out of cells, and were shown to play an important role in the intracellular antiviral drugs concentration [12–17]. The genes coding for these transport proteins are polymorphic in humans, with consequences in term of expression and function, potentially influencing the intracellular levels of antiretroviral drugs.

Thus, as intracellular concentrations of antiretroviral drugs are influenced by both their physico-chemical properties and host genetic factors, an assay enabling the monitoring of PIs and NNRTIs levels at the site of their pharmacological action appears to be an essential tool for the ongoing investigations aimed at preventing antiretroviral therapy failure or toxicity. Owing to its sensitivity and selectivity, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is particularly attractive for the measurement of intracellular analytes [18]. LC–MS/MS technology has been applied for measuring the active triphosphate anabolites of several nucleosidic reverse transcriptase inhibitors (NRTIs) in PBMCs [19–22]. PIs and NNRTIs are directly acting pharmacological species requiring no bioactivation but, although several LC–MS/MS assays have been published for the quantitative determination of PIs in plasma [23–28], validated methods for their determination in PBMCs have rarely been described. Except two reports recently published for atazanavir [29] and for four PIs simultaneously measured with efavirenz [30], the analytical methods for the intracellular measurements of PIs and NNRTIs have been published up to now only in excerpt form, as part of *Materials and methods* section [31–33], with limited information on method validation procedures. An enzyme immunoassay for nevirapine plasma and intracellular levels measurement has been published recently [34].

In this report, we describe the development and validation of an analytical method for the quantification in PBMCs of nine antiretroviral drugs (indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz, nevirapine), by liquid chromatography coupled with tandem triple quadrupoles mass spectrometry detection. This method is characterized by a very low limit of quantification ($\leq$0.5 ng/ml), well below the clinically relevant range of concentration encountered in patients PBMCs contained in a 8 ml-blood cell preparation tube (CPT, Vacutainer®). The stringent workup for PBMCs isolation and careful washing is counterbalanced by a simplified extraction step. Since experience in handling PBMCs samples for intracellular drug measurements are rather limited, special attention has been given to describe in detail the methodology we used.

2. Experimental

2.1. Chemicals and reagents

Ritonavir (RTV), lopinavir (LPV), saquinavir (SQV), nelfinavir (NFV), amprenavir (APV), indinavir (IDV), atazanavir (ATV), efavirenz (EFV) and nevirapine (NVP) (Figs. 1 and 2) were kindly provided by Abbott (Baar, Switzerland), Roche discovery Welwyn (Welwyn Garden City, UK), Agouron (La Jolla, CA, USA), Glaxo Wellcome R&D (Stevenhage, UK), Bristol Myers Squibb (Baar, Switzerland), Merck Sharp Dohme Chibret (Glattburg, Switzerland) and Boehringer Ingelheim (Ridgefield, CT, USA), respectively. Clozapine (internal standard, I.S.) stock solution (250 μg/ml) in MeOH was obtained by extraction with MeOH of Leponex® (Novartis, Basel, Switzerland) tablet. Solvent used for chromatography such as acetonitrile (MeCN) and methanol (MeOH), both of LiChrosolv® grade, and 100% formic acid were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). The phosphate buffered saline (PBS) solution used for the preparation of PBMCs matrix was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the heat inactivated foetal bovine serum (FBS) from Invitrogen (Basel, Switzerland).

Blank PBMCs used for the assessment of matrix effect and for the preparation of calibration and control samples were isolated from leucocytes collected in filters (ASAHI Sepacell RZ 2000, Baxter, La Châtre, France) routinely employed for the leukapheresis of blood donation units in the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland). Isolation of PBMCs from leucocytes was carried out using a Ficoll-Hypaque density gradient centrifugation solu-