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Validation and clinical application of a high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of 10 anti-retrovirals in human peripheral blood mononuclear cells

L. Elens [a,*], S. Veriter [a], J.C. Yombi [b], V. Di Fazio [c], R. Vanbinst [c], D. Lison [a], P. Wallemacq [a,c], B. Vandercam [b], V. Haufroid [a,c]

[a] *Louvain center for Toxicology and Applied Pharmacology (LTAP), Université catholique de Louvain, Avenue E. Mounier, 53.02, 1200 Brussels, Belgium*
[b] *Department of Internal Medicine, Unit of Infectious Diseases, St-Luc University Hospital, Brussels, Belgium*
[c] *Clinical Chemistry Department, St-Luc University Hospital, Brussels, Belgium*

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ABSTRACT

This paper reports the validation of a liquid chromatography tandem mass spectrometry (LC-MS/MS) method that allows the quantification of 10 antiretroviral (ARV) drugs in peripheral blood mononuclear cells (PBMCs) using 6 different isotopic internal standards (IS) and its clinical application. PBMCs are isolated from blood by density gradient centrifugation and drugs are extracted with a 60% methanol (MeOH) solution containing the 6 IS. The cell extract is then injected in the HPLC system and analytes are separated on a Symmetry Shield RP18 2.1 mm × 50 mm column. The different molecules are then detected by MS/MS in electrospray positive or negative ionisation modes and data are recorded using the multiple reaction monitoring (MRM) mode. Calibration curves are constructed in the range of 0.25–125 ng/ml of cell extract by a $1/x^2$ weighted quadratic regression. The regression coefficients obtained are always greater than 0.99 and back calculated values always comprised in the range of ±15% from their nominal concentration. Mean extraction recoveries are greater than 80% for all analytes and the method is accurate and precise with CV and bias lower than 9.4%. The lower limits of quantification (LLOQ) of the different drugs range from 0.0125 to 0.2 ng/ml of cell extract. This method was successfully applied to a cohort of 98 HIV-infected patients treated with Kaletra® (400/100 mg of lopinavir/ritonavir (LPV/RTV) twice a day, $n = 48$) or with Stocrin® (600 mg once a day, $n = 50$) and has been tested for cellular quantification of tipranavir (TPV) in 2 patients treated with Aptivus® (500 mg twice a day). The patients treated by Kaletra® showed mean cell-associated concentrations (CC) of 1819.0 and 917.2 ng/ml, for LPV and RTV, respectively. Patients treated with Stocrin® showed mean CC of 2388.11 ng/ml while both patients under Aptivus® showed TPV CC of 4322.7 and 1078.0 ng/ml, respectively. This method can be used to analyze ARV drug concentrations within the target tissue.

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1. Introduction

Analytical tools for monitoring the plasma concentrations of anti-HIV drugs are largely available in clinical settings and have already contributed to improve anti-HIV therapy through the means of therapeutic drug monitoring (TDM). Despite these advances, failures of anti-HIV therapy are still frequently encountered in daily clinical practice and the reasons for these failures are not always understood. A possible explanation could be that measuring plasma drug concentration is of limited clinical value because the major target of these drugs is within the infected cells and only the fraction reaching this intracellular compartment is expected to be active against HIV replication. In this respect, several studies have, however, shown a good correlation between intracellular and plasma drug concentrations for several protease inhibitors (PIs) [1–3] but not for NNRTIs [3,4], suggesting that plasma concentration could constitute a valid parameter for the TDM of PIs but not, or at least less accurately, for NNRTIs. Direct measurement of intracellular drug concentrations may therefore contribute to improve and adjust antiretroviral therapy. A method to quantify the active fraction of these drugs would allow to better characterize the pharmacokinetics (PK) and the accumulation profile of these drugs in the cellular compartment and, would possibly give a better insight into understanding the reasons for some therapeutic

* Corresponding author. Tel.: +32 2 764 53 54; fax: +32 2 764 53 38.
E-mail address: laure.elens@uclouvain.be (L. Elens).

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failures. Also, it would allow to investigate the determinants of the variability in intracellular concentrations and the potential discrepancies between plasma and intracellular concentrations.

To date, only few LC-MS/MS methods are fully validated for the intracellular quantification of PIs and/or NNRTIs [5–9], in comparison to the number of validated LC-MS/MS methods available for the quantitative determination of PIs and NNRTIs in plasma [10–21]. Most intracellular quantification methods are only partially described in clinical reports [1,2,22–27]. Furthermore, among fully validated methods, only two allow the simultaneous intracellular quantification of all commercially available PIs and NNRTIs, except tipranavir [5] and atazanavir [8].

The present paper reports an improved LC-MS/MS method using isotopic internal standards allowing the intracellular quantification of the relatively new PI, tipranavir (TPV).

2. Experimental

2.1. Chemicals, biologicals and reagents

The drugs investigated were amprenavir (APV), atazanavir (ATZ), efavirenz (EFV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), nevirapine (NVP), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV). APV and ATZ were kindly provided by GlaxoSmith Kline Research and development (Middelsex, UK) and Bristol-Myers and Squibb (New Brunswick, NJ, USA), respectively. NFV and SQV were kindly provided by Roche diagnostics (Mannheim, Deutschland), and IDV and EFV by Merck (NJ, USA). LPV and RTV were kindly provided by Abbott Laboratories (Chicago, USA). NVP and TPV were kindly provided by Boehringer and Ingelheim (Ridgefield, USA). IDV-d6 and SQV-d5 were kindly provided by Merck and Roche diagnostics, respectively. Other isotopic internal standards (EFV-d4, LPV-d8, ATZ-d5 and RTV-^{13}C13) were purchased from Toronto Research Chemicals (North York, Ontario, Canada).

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate and acetic acid were obtained from Merck (Germany), formic acid from Sigma–Aldrich (Germany) and ficoll-Paque™ Plus solution from Amersham Biosciences AB (Uppsala, Sweden). The Dulbecco's phosphate buffered saline (DPBS) solution and foetal bovine serum (FBS) were obtained from Invitrogen.

Blank PBMCs used for calibration curves and quality control (QC) samples and for the assessment of matrix effect were isolated from leucodepletion filters (Leucoflex LCR, Macopharma) kindly provided by the blood transfusion centre of Namur (Belgium).

PBMCs count was performed on a Sysmex K-1000 haematology analyzer (Norderstedt, Germany).

2.2. LC-MS/MS system and chromatographic conditions

The HPLC system consisted of a Waters 2795 Alliance High Throughput HPLC system with an integrated autosampler (Waters, Mildford, MA, USA) thermostated at 10 °C. The chromatographic separation was performed on a Symmetry shield RP18, 2.1 mm × 50 mm column (Waters) applied in an oven maintained at 25 °C. The chromatographic system was coupled with a Quattro *micro*™ tandem mass spectrometer (Micromass UK Ltd., Manchester, UK) fitted with a Z-Spray™ ion source. The instrument was operated in both electrospray positive and negative ionisation modes. All aspects of system operation and data acquisition were controlled by a *MassLynx NT*™ v3.5 software (Micromass, Manchester, UK) and data processing was performed with the *QuanLynx*™ Application Manager (Micromass). Data were recorded in the multiple reaction monitoring (MRM) mode.

The mobile phase was delivered in the column with a start flow rate of 0.3 ml/min. Eluent A consisted of 10 mM ammonium acetate/10 mM formic acid and eluent B was ACN/10 mM formic acid. From 0 to 2 min, the mobile phase consisted of 95% of eluent A and 5% of B. A linear elution gradient was set to reach 10% of A and 90% of B at 12 min followed by an accelerated rinsing with 100% of B at a flow rate of 0.5 ml/min. from 12 to 15 min and of 0.4 ml/min till 18 min. Initial conditions were restored in 2 min and maintained during 5 min for re-equilibration.

The mass spectrometer was operated in electrospray positive and negative ionisation modes for PI/NVP and EFV, respectively. Consequently, the mass of the precursor ions corresponded to M + 1 and M − 1 for PI/NVP and EFV, respectively. Tuning of the MS/MS detector was performed by direct injection of compounds with a syringe at concentration of 1000 ng/ml in 50:50 ACN/H_2O (10 mM formic Acid). Optimized detection parameters are reported in Table 1. Three distinct windows of acquisition were programmed in the positive mode and one in the negative mode. The source and capillary temperature were maintained at 120 and 300 °C, respectively, while the capillary voltage was set at 3.5 kV. The collision gas was argon, and pressure was monitored at 2.5×10^{-6} bar in the collision cell. The nebulising, and cone gas was nitrogen and set at flow rates of 550 and 20 l/h, respectively.

2.3. Stock solutions, calibrators and quality controls (QC)

All drugs and internal standards were solubilised in methanol at a concentration of 1 mg/ml (stock solution) and kept at −80 °C. A working solution of the six internal standards at a concentration of 4000 ng/ml was prepared in 60:40 MeOH:H_2O. Similarly, a working solution containing all drugs at a concentration of 1,250 ng/ml was prepared in 60:40 MeOH:H_2O by appropriately diluting the stock solutions.

These working solutions were then diluted in 60:40 MeOH:H_2O to obtain 8 calibrators (0.25; 0.625; 1.25; 6.25; 12.5; 62.5; 93.75; 125 ng/ml for all drugs) and 3 QC solutions (2.5, 25 and 75 ng/ml) containing all 6 IS (IDV-d6, SQV-d5, EFV-d4, LPV-d8, ATZ-d5 and RTV-^{13}C13) at a concentration of 20 ng/ml. The extraction solvent consisted of a 60:40 MeOH:H_2O solution containing the 6 IS at 20 ng/ml.

2.4. Blank and patients PBMCs preparation and drug extraction procedure

2.4.1. Preparation of blank PBMCs isolated from leucodepletion filters

This method was adapted from [5] with slight adaptations. Leucodepletion filters were washed with 30 ml DPBS supplemented with 2% FBS. PBMCs were then isolated from total leucocytes population by Ficoll density gradient separation (Ficoll-Paque™ Plus). Three washing steps were performed with DPBS solution. PBMCs were quantified by cell counting on a burker cell with Trypan blue coloration and then, aliquoted in 1.5 ml Eppendorf to obtain typically between 3 and 8×10^6 cells/vial. Finally, the vials were centrifuged at 650 × *g*, the supernatant was discarded and PBMCs were immediately stored at −20 °C.

2.4.2. Collection of PBMCs from patients

The patients were recruited at the Saint-Luc Hospital (Brussels) and the samples were drawn together with those collected for routine clinical follow-up. The protocol of the present study has been approved by the local ethical committee and written informed consent was obtained from each patient. 48 patients receiving Kaletra® (LPV/RTV 400/100 mg twice daily) associated with 2 NRTIs (3TC + AZT $n = 7$; 3TC + ABC $n = 23$; 3TC + d4T $n = 1$) or tenofovir plus one NRTI (3TC $n = 15$; emtricitabine $n = 1$) or 3NRTIs (3TC + AZT + d4T $n = 1$) were recruited for the present study between July 2007 and January 2008. These patients did not receive another

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