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Simultaneous quantification of intracellular lamivudine and abacavir triphosphate metabolites by LC–MS/MS

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) require intracellular phosphorylation to active triphosphate (TP) nucleotide metabolites before they can inhibit the HIV reverse transcriptase. However, monitoring these pharmacologically active TP metabolites is challenging due to their instability and their low concentrations at the pg/ml levels in blood and tissues. The combination of lamivudine (3TC) and abacavir (ABC) is one of the first lines for HIV therapy. Therefore, a sensitive, selective, accurate, and precise LC–MS/MS method was developed and validated for the simultaneous quantification of 3TC- and ABC-TP metabolites in mouse blood and tissues. Calibration curves were linear over the range of 10–100,000 pg/ml for 3TC-TP and 4–40,000 pg/ml for carbovir-TP (CBV-TP; phosphorylated metabolite of ABC). This corresponds to 2.1–21,322 fmol/10^6 cells for 3TC-TP and 0.8–8000 fmol/10^6 cells for CBV-TP. Accuracy and precision were less than 15% for all quality control sample (QCs), and absolute extraction recovery of were >65% for 3TC-TP and >90% for CBV-TP. The method was optimized to ensure stability of TP samples and standards during sample collection, preparation, analysis, and storage conditions. This method has enhanced sensitivity and requires smaller amounts of blood and tissue samples compared to previous LC–MS/MS methods for 3TC- and CBV-TP quantification. The developed method was successfully applied to characterize the pharmacokinetic profile of TP metabolites in mouse peripheral blood mononuclear cells (PBMCs), spleen, lymph nodes, and liver cells. In addition, another direct, simple, and high-throughput method for the quantification of TP standards was developed and used for the analysis of stability samples.

1. Introduction

The Department of Health and Human Services (DHHS) guidelines for antiretroviral drug administration in human immunodeficiency virus type one (HIV-1)-infected adults and adolescents recommends the use of two nucleoside reverse transcriptase inhibitors (NRTIs) in combination with a non-nucleoside reverse transcriptase (NNRTI), an integrase strand transfer (INSTI), or a protease inhibitor (PI). For the former, NRTIs are prodrugs that require intracellular phosphorylations to active triphosphate (TP) nucleotide metabolites, which are responsible for the inhibition of the viral reverse transcriptase [1,2]. NRTIs are converted intracellularly to mono-, di-, and then tri- phosphates by nucleoside phosphate kinases. The rate determining step in this 3-step enzymatic conversion to TP metabolites is the first step of parent nucleoside phosphorylation into monophosphate metabolites [3,4]. Lamivudine (3TC) and abacavir (ABC) are amongst the most commonly used NRTIs [5–7] and are commercially available as a combination in a single product.

Similar to all NRTIs, 3TC and ABC are metabolized intracellularly to their active TP metabolites. However, during phosphorylation, ABC is also deaminated into carbovir (CBV). ABC is converted to ABC-5′-monophosphate (ABC-MP) by an adenosine phosphotransferase and subsequently deaminated into carbovir 5′-monophosphate (CBV-MP) by a cytosolic deaminase. CBV-MP is then further phosphorylated in two successive steps to the active metabolite CBV-5′-triphosphate(CBV-TP) [1].

Monitoring NRTI intracellular TP metabolites, which exist at the pg/ml levels, is important to understand their pharmacological effects [8,9]. Therefore, very sensitive and selective methods

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are required for their quantification. Mass spectrometry coupled with ultra-performance liquid chromatography (UPLC–MS/MS) is the gold standard technique for the quantification of NRTIs and their phosphorylated nucleotide metabolites.

Nucleotides contain 1–3 phosphate groups, deoxyribose or ribose sugar, and a purine or a pyrimidine nitrogen base [10]. Due to their hydrophilicity, chromatographic separation of nucleotides is challenging using traditional reverse phase chromatography. Therefore, alternative approaches are used for the chromatographic separation of nucleotide analytes, which can be grouped into two categories, direct and indirect approaches [8]. Direct liquid chromatography (LC)-MS methods rely on the direct quantification of the nucleotide metabolites under non-reverse phase LC conditions. Whereas, indirect methods rely on the quantification of the parent nucleosides resulting from the dephosphorylation of nucleotide metabolites during sample preparation, under reverse phase LC conditions. Both approaches have their advantages and disadvantages.

Several LC–MS/MS methods are available for the quantification of 3TC or ABC, and their triphosphate metabolites [11–20]. However, these methods were developed for the quantification of TPs in peripheral blood mononuclear cells (PBMCs) obtained from relatively large volumes of blood in humans. In this study, we developed, validated, and applied two sensitive and selective LC–MS/MS methods for quantification of these TP metabolites in PBMCs obtained from smaller blood volumes in mice. In addition, we preformed quantitation of TP metabolites in mouse immune cells obtained from spleen, liver, and lymph nodes. This method will be used to support the preclinical development of NRTIs as long-acting nanoformulated antiretroviral therapies.

2. Materials and methods

2.1. Materials

3TC-TP, CBV-TP, CBV, $^{15}N_2{}^{13}C$-3TC, d_4-ABC, and emtricitabine-TP (FTC-TP) were obtained from Toronto Research Chemicals (North York, Canada). 3TC and ABC were provided by GlaxoSmithKline Inc. (Research Triangle Park, NC). Type XA acid phosphatase (EC 3.1.3.2) from sweet potatoes, guanosine, guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), cytidine, cytidine monophosphate (CMP), cytidine diphosphate (CDP), and cytidine triphosphate (CTP) were purchased from Sigma Chemical Co. (St. Louis, MO). Sep-Pak QMA anion- exchange cartridges and OASIS HLB reverse-phase cartridges were purchased from Waters Corp. (Milford, MA). Potassium chloride (KCl) was purchased from JT Baker Co. (Phillipsburg, NJ). LC–MS grade (Optima) water (H_2O), acetonitrile (ACN), methanol (MeOH), acetic acid, ammonium hydroxide, and ammonium bicarbonate were purchased from Fisher chemicals (Fair Lawn, NJ). RPMI media 1640, phosphate buffered saline (PBS), Hanks' Balanced salt solution (HBSS), and fetal bovine serum were purchased from Invitrogen (Gibco; Carlsbad, CA).

2.2. Instrumentation

2.2.1. LC–MS/MS conditions for the indirect quantification of TP metabolites

The LC–MS/MS system comprised of a Waters ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to a triple quadrupole mass spectrometer with electrospray ionization (ESI) source (Waters Xevo TQ-XS). For the indirect quantification of TPs, chromatographic separation was performed with an ACQUITY UPLC using a CSH C18 analytical column (2.1 × 100 mm, 1.7 μm; Waters) equipped with a guard column (Waters, Milford, MA). Mobile phase A consisted of ammonium bicarbonate (pH 7, 7.5 mM) and mobile phase B was methanol. The flow rate was 0.25 ml/min. The initial mobile phase composition was 12% B for the first 2.5 min, gradually increased to 30% B over 4 min, gradually increased again to 95% B over 3.5 min, and then held constant for one min. Mobile phase B was then reset to 12% over 0.25 min and the column was equilibrated for 2.75 min before the next injection. The total run time was 13 min. The mass spectrometer was operated in the positive ion mode using multiple reaction monitoring (MRM). The following transitions were monitored: m/z 230 → 112 for 3TC, m/z 248 → 152 for CBV, m/z 287 → 191 for ABC, m/z 233 → 115 for the internal standard (IS) $^{15}N_2{}^{13}C$-3TC, and m/z 291 → 195 for IS d_4-ABC. 3TC, CBV, ABC, $^{15}N_2{}^{13}C$-3TC, and d_4-ABC were detected at a cone voltage of 22, 2, 4, 12, and 2 V, respectively, and a collision energy of 12, 12, 20, 10, and 20 V, respectively.

2.2.2. LC–MS/MS conditions for direct quantification of TP metabolites

For direct quantitation of TP metabolites, the LC–MS/MS system comprised of a Waters ACQUITY-UPLC system coupled to a 4000 Q TRAP® quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, MDS Sciex, Foster City, CA). The chromatographic separation was performed with an anion-exchange column BioBasic-AX (150 mm × 2.1 mm, 5 μm; Thermo Fisher, Waltham, MA). Mobile phase A consisted of 40% ACN, 0.06% acetic acid, and 10 mM ammonium formate in water, and mobile phase B comprised of 30% ACN, 0.3% ammonium hydroxide, and 1 mM ammonium formate in deionized water. The flow rate was 0.45 ml/min. The initial mobile phase composition was 50% B for the first min, gradually increased to 90% B over two min, and then held constant for five min. Mobile phase B was then reset to 50% over 0.25 min and the column was equilibrated for 1.75 min before the next injection. The total run time was 10 min. The mass spectrometer was operated in the positive ion mode using multiple reaction monitoring (MRM). The following transitions were monitored: m/z 470 → 112 for 3TC-TP, m/z 488 → 152 for CBV-TP, and m/z 488 → 130 for the IS (FTC-TP). 3TC-TP, CBV-TP, and FTC-TP were detected at a declustering potential (DP) of 66 V, 106 V, and 96 V, respectively, and a collision energy of 45, 43, and 25 V, respectively.

2.3. Sample preparation

2.3.1. Extraction of intracellular TP metabolite for the indirect TP method

Sep-Pak QMA cartridges (360 mg, 37–55 μm; Waters) were used to separate CBV-TP, and 3TC-TP from their mono- and di-phosphates counterparts. The QMA cartridges were conditioned with 10 ml of 500 mM KCl followed by 10 ml of 5 mM KCl. Cell lysate samples in 200 μl of 70% MeOH were loaded onto the cartridges and washed with 15 ml of 75 mM KCl. The triphosphate fraction was eluted with 3 ml of 500 mM KCl and collected for de-phosphorylation. The pH of the TP fraction was lowered to 4.25 by adding 15 μl ammonium acetate buffer (pH 4,10 mM) per ml eluate, and dephosphorylated by adding one unit of type XA sweet potato acid phosphatase per ml eluate and incubating at 37 °C for 30 min The $^{15}N_2{}^{13}C$-3TC internal standard was added at this point (10 μl of 5 ng/ml, final concentration of 0.5 ng/ml). Samples were then loaded onto Waters OASIS HLB cartridges (60 mg, 30 μm; Waters) pre-conditioned with 3 ml MeOH and 3 ml H_2O, and washed with 3.5 ml H_2O to remove salts. The nucleosides of interest were then eluted with 1.5 ml of MeOH and evaporated under vacuum. Once dry, the residue was reconstituted with a 100 μl of 25% MeOH and stored in the −20 °C freezer until the time of LC–MS/MS analyses.