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High throughput LC–MS/MS method for simultaneous determination of tenofovir, lamivudine and nevirapine in human plasma

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ABSTRACT

A selective and high throughput liquid chromatography–mass spectrometry method has been developed and validated for the simultaneous quantification of tenofovir (TFV), lamivudine (3TC) and nevirapine (NVP) in human plasma using emtricitabine (FTC) as internal standard (ISTD). Following solid phase extraction (SPE), the analytes and ISTD were run on Prontosil C18AQ column (100 mm × 4.6 mm, 3 μm) using an isocratic mobile phase consisting of 1 mM ammonium acetate in water (pH 6.5 ± 0.3):acetonitrile (50:50, v/v). The precursor and product ions of the drugs were monitored on a triple quadrupole instrument operated in the negative ionization mode. The method was validated over a concentration range of 2–500 ng/mL for TFV and over a concentration range of 10–4000 ng/mL for 3TC and NVP with relative recoveries ranging from 61 to 85%. The intra and inter batch precision (%CV) across four validation runs was less than 12.2%. The accuracy determined at four QC levels (LLOQ, LQC, MQC and HQC) was within ±8.5%, in terms of relative error.

1. Introduction

Introduction of multi-class drug combination treatment [1] for acquired immuno deficiency syndrome (AIDS) [2] has become great success in the history of modern medicine. In a span of 15 years mortality rate in HIV infected patients was reduced by 50–80% and currently the disease is considered to be a controllable chronic illness. Due to increased resistance of the causative human immunodeficiency virus (HIV) [3], US Department of Health and Human Services has recommended highly effective single and multiple class combination drug regimens which are often called as highly active antiretroviral therapy (HAART) [4]. The most common combination at the beginning of treatment consists of two nucleoside reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). NRTIs interfere intracellularly with the reverse transcriptase enzyme of HIV, which is essential for viral replication. While the NNRTIs stop HIV from replicating within human cells by non-competitive inhibition of the reverse transcriptase [5–8]. PIs inhibit protease, which is another enzyme involved in the HIV replication process. Synergistic activity of antiretroviral combinations has improved the quality of AIDS treatment along with increased patient compliance.

Tenofovir (TFV) is a potent nucleotide analog generally administered as its pro drug tenofovir disproxil fumarate (TDF) [9]. TFV inhibits the activity of HIV reverse transcriptase by competing with deoxyadenosine 5′-triphosphate and, after incorporation into DNA, by DNA chain termination [10]. TFV has low protein binding and its oral bioavailability is approximately 25%. Lamivudine (3TC) a nucleoside reverse transcriptase inhibitor is active against HIV type-1 and hepatitis B (HBV) [11]. Intracellularly 3TC phosphorylate to form active 5′-triphosphate metabolite, lamivudine triphosphate. It competitively inhibits the HIV reverse transcriptase enzyme and acts as chain terminator of DNA synthesis. On oral administration, absorption of 3TC is rapid and absolute bioavailability is approximately 86% for both tablet and oral solution. Nevirapine (NVP) is a potent non-nucleoside reverse transcriptase inhibitor used in combination with nucleoside analogs for HIV infection [12]. It is active against HIV type-1 and in general prescribed after the immune system has declined and infections have become more evident. NVP binds directly to reverse transcriptase and blocks the RNA dependent and DNA dependent DNA polymerase activities by causing disruption in the enzyme catalytic site. On oral administration, its systemic availability is about 90% with a relative longer half-life of 45 h.

TFV, 3TC and NVP combination is one of the four recommended regimens by WHO for antiretroviral therapy in resource-limited

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settings, and is supplied as fixed dose combination of TFV and 3TC co-packaged with NVP. The combination is proven to be cost effective in first-line treatment with appropriate therapeutic activity [13]. Several analytical methods [14–29] were reported for the determination of TFV, 3TC and NVP individually or in other combinations in pharmaceutical formulations and in biological matrices, however no method was available for the simultaneous determination of TFV, 3TC and NVP in human plasma. Lanka et al. [14] developed a stability indicating HPLC method for the determination of TFV, 3TC and NVP in tablet dosage forms. Matta et al. [19] developed a LC–MS/MS method for the simultaneous determination of TFV and 3TC in human plasma with a LOQ of 5 ng/mL for TFV and 25 ng/mL for 3TC. Yadav et al. [21] developed a simultaneous LC–MS/MS method for the determination of TFV, 3TC and FTC in human plasma with a LOQ of 4 ng/mL for TFV and 20 ng/mL for 3TC.

Aim of the current study is to develop and validate [30–33] a sensitive and high throughput method for the simultaneous determination of TFV, 3TC and NVP in human plasma for therapeutic drug monitoring and pharmacokinetic studies. As a part of it developed and validated an isocratic LC–MS/MS method with simple and reproducible SPE procedure using FTC as ISTD. The developed method was successfully used to study the pharmacokinetics of TFV and 3TC tablets (300 + 300 mg), in healthy human volunteers under fasting condition.

2. Experimental

2.1. Chemicals and reagents

Working standards of tenofovir (TFV), lamivudine (3TC), nevirapine (NVP) and emtricitabine (FTC) having purity more than 99% were obtained from Aurobindo Pharma Ltd. (Hyderabad, India). LC–MS grade methanol and acetonitrile were purchased from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). GR grade ammonium acetate and ammonia solution (30% pure) were procured from Merck Specialties Pvt. Ltd. (Mumbai, India). GR grade orthophosphoric acid was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). HPLC type I water was obtained from Milli-Q A10 gradient water purification system (Millipore, Bedford, MA, USA). Drug free human plasma containing K_2 EDTA anticoagulant was obtained from Doctor's pathological lab (Hyderabad, India). MCX 30 mg, 1 CC solid phase extraction cartridges were purchased from Waters Corporation (Milford, MA, USA).

2.2. Instrumentation

Modular HPLC system (Shimadzu, Kyoto, Japan) consisting of binary LC-20AD prominence pumps, DGU-20 A3 solvent degasser, CTO-AS vp column oven and high throughput SIL HT_C autosampler was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ionspray interface. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software version 1.4.2 (SCIEX) was used for controlling the hardware and data handling.

2.3. Chromatographic conditions

Chromatographic separation was performed on Prontosil C18AQ, 100 mm × 4.6 mm, 3 μm analytical column (Bischoff chromatography, Germany). Isocratic mobile phase consisting of 1 mM ammonium acetate buffer in water (pH 6.5 ± 0.3):acetonitrile (50:50, v/v) was delivered at a flow rate of 1.0 mL/min. The autosampler was set at 10 °C and the injection volume was 10 μL. The column oven temperature was maintained at 35 °C. The total chromatographic run time was 3.0 min.

2.4. Mass spectrometric conditions

Turbo ionspray interface (TIS) operating in the negative ionization mode was used to study the parent → product ion (m/z) transitions for TFV (286.1 → 134.0), 3TC (228.1 → 134.0), NVP (265.0 → 181.9) and FTC (246.0 → 152.0). Declustering potential (DP), entrance potential (EP), collision energy (CE), collision exit potential (CXP) were all optimized to allow the highest possible signal transduction with low background noise. Signal optimization was performed by constant infusion of 100 ng/mL drug solutions in 50% acetonitrile at a rate of 50 μL/min. The pressure of the drying gas was 35 psi and the temperature was 350 °C. The ionspray voltage was set at −4500 V and the pressure of collision gas (nitrogen) was 4 psi. The quadrupoles 1 and 3 were set at unit mass resolution and each MRM transition was monitored with a dwell time of 200 ms.

2.5. Preparation of calibration standards and quality control samples

Standard stock solutions of 3TC, NVP and internal standard (FTC) were prepared by dissolving their accurately weighed amounts in methanol while TFV was dissolved in milli-Q water to give a final concentration of 1000 μg/mL. Individual working solutions of analytes were prepared by appropriate dilution of their stock solutions in 50% acetonitrile. All the solutions were stored in refrigerator at below 10 °C and were brought to room temperature before use. Working solution of internal standard (FTC, 500 ng/mL) was prepared daily in 50% acetonitrile and was stored at room temperature.

Calibration standards and quality control (QC) samples were prepared by spiking (3%) blank plasma with the working solutions prepared from independent stock weightings. K_2 EDTA anticoagulant blank plasma collected from healthy, non-smoking volunteers was screened individually and pooled before use. Calibration standards were prepared at concentrations of 2, 4, 10, 40, 100, 200, 400, 500 ng/mL for TFV and at 10, 20, 50, 200, 500, 2000, 3200, 4000 ng/mL for 3TC and NVP.

Quality control samples were prepared at 2 ng/mL (LLOQ QC), 5.9 ng/mL (LQC), 200 ng/mL (MQC) and 380 ng/mL (HQC) for TFV; at 10 ng/mL (LLOQ QC), 29.9 ng/mL (LQC), 1680 ng/mL (MQC) and 3050 ng/mL (HQC) for 3TC and NVP (LLOQ QC was prepared only for validation batch runs). 0.3 mL aliquots of spiked plasma samples were taken in polypropylene tubes and stored at −70 °C. Prior to analysis all frozen subject samples, calibration standards and quality control samples were thawed unassisted at room temperature and subjected to the sample preparation procedure as indicated in Section 2.6.

2.6. Sample preparation

Samples of 0.2 mL of plasma were dispensed into 5 mL polypropylene tubes and added 50 μL of ISTD solution (FTC, 500 ng/mL), followed by 200 μL of 2.0% orthophosphoric acid in water. The resulting samples were vortex mixed and then subjected to the SPE procedure using MCX 30 mg/1 CC cartridges on a positive pressure SPE unit. Before the samples were loaded, the SPE columns were sequentially conditioned with 1 mL of methanol and 1 mL of 0.5% orthophosphoric acid in water. The columns were washed sequentially with 1 mL of 0.5% orthophosphoric acid in water and 1 mL of 5% methanol in water. The elution was performed with 1 mL of 5% ammonia in methanol. The eluants were dried in a stream of nitrogen at 50 °C in a Turbovap apparatus (Zymark, MA, USA) for 15 min. The dried samples were reconstituted with