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ORIGINAL ARTICLE

Selective and rapid determination of raltegravir in human plasma by liquid chromatography–tandem mass spectrometry in the negative ionization mode

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Abstract A selective and rapid high-performance liquid chromatography–tandem mass spectrometry method was developed and validated for the quantification of raltegravir using raltegravir-d3 as an internal standard (IS). The analyte and IS were extracted with methylene chloride and n-hexane solvent mixture from 100 μL human plasma. The chromatographic separation was achieved on a Chromolith RP-18e endcapped C_{18} (100 mm × 4.6 mm) column in a run time of 2.0 min. Quantitation was performed in the negative ionization mode using the transitions of *m/z* 443.1 → 316.1 for raltegravir and *m/z* 446.1 → 319.0 for IS. The linearity of the method was established in the concentration range of 2.0–6000 ng/mL. The mean extraction recovery for raltegravir and IS was 92.6% and 91.8%, respectively, and the IS-normalized matrix factors for raltegravir ranged from 0.992 to 0.999. The application of this method was demonstrated by a bioequivalence study on 18 healthy subjects.

1. Introduction

Raltegravir (RAL), a hydroxypyrimidinone carboxamide derivative, is an integrase strand-transfer inhibitor (INSTI) used in the treatment and management of human immunodeficiency virus (HIV) infection [1]. It was first approved by USFDA in 2007 for

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the treatment of HIV treatment-experienced patients [2]. RAL is considered as the first generation INSTI that has demonstrated considerable efficacy in the treatment of naive as well as HIV treatment-experienced adult patients with viral resistance. It inhibits the catalytic activity of HIV-1 integrase enzyme, which is responsible for viral replication by blocking the viral DNA into the cellular genome by binding to the integrase-viral DNA complex [3–5]. RAL is approximately 83% plasma bound and gets rapidly absorbed from the gastrointestinal tract, with peak plasma concentration achieved within 0.5–1.3 h. It undergoes hepatic metabolism mainly by uridine diphosphate glucuronosyltranferase enzyme to give an inactive glucuronide metabolite, with only 9% of the administered dose excreted unchanged in the urine [6]. Further, the pharmacokinetics of RAL showed important inter- or intra-subject variability [7].

Selective and sensitive determination of anti-HIVs in plasma is essential for studying drug–drug interaction, pharmacokinetics, pharmacodynamics and therapeutic drug monitoring. Several methods are reported for the determination of RAL as a single analyte [8–20] or in combination with its glucuronide (Glu) metabolite [21] or other anti-HIV drugs [22–26] in different biological samples such as human cell extracts [15,19], cerebrospinal fluid [16], cervicovaginal fluid [17], dried blood spots [15], bile [9], feces [9] and human plasma [8,10–14,18,20–26]. Mainly, liquid chromatography with UV [10,17,23], photodiode array [18,22], fluorescence [11] or mass spectrometry [8,9,12–16,19–21,24–26] detection has been used for the quantification of RAL in these matrices.

All reported methodologies using LC–MS/MS quantified RAL in the positive ionization mode and few discussed in-source conversion of RAL-Glu to RAL [14,15,21,24] and its possible interference in the quantification of RAL. Thus, it is essential to develop an adequately sensitive, rapid and selective method, with chromatographic resolution of interfering compounds like RAL-Glu to avoid overestimation of RAL concentration. In the present work, negative ionization mode was selected as it showed better selectivity without compromising the sensitivity of the method. Further, the chromatographic conditions were suitably optimized on a Chromolith RP-18e endcapped C_{18} column under isocratic elution for baseline separation of RAL from RAL-Glu. The method described was used to support a bioequivalence study in healthy Indian subjects.

2. Experimental

2.1. Chemicals and materials

Reference standard of raltegravir (98.5%) was procured from Hetero Drugs Limited (Hyderabad, India). Raltegravir-d3 (IS, 98%) and raltegravir glucuronide were procured from Toronto Research Chemicals Inc. (Ontario, Canada). HPLC grade methanol, acetonitrile, ammonium formate, dichloromethane (DCM), *n*-hexane and formic acid were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

2.2. Liquid chromatographic and mass spectrometric conditions

An LC-VP HPLC system (Shimadzu, Kyoto, Japan) was used for chromatographic separation of RAL and IS on Chromolith RP-18e endcapped C_{18} (100 mm × 4.6 mm) analytical column having monolithic silica rod (Phenomenex, Hyderabad, India) maintained at 40 °C in the column oven. For isocratic elution, the mobile phase consisting of 10 mM ammonium formate in water, pH 3.0, and acetonitrile (30:70, v/v) was delivered at a flow-rate of 1.2 mL/min. The total eluate from the column was split in 85:15 (v/v) ratio; flow directed to the electrospray interface was equivalent to 180 µL/min. The autosampler temperature was maintained at 5 °C and the average pressure of the system was 1200 psi. A triple quadrupole mass spectrometer API-4000 (AB/MDS SCIEX, Toronto, Canada) equipped with electrospray ionization and operating in negative ionization mode was used for detection of RAL and IS. For quantitation, multiple reaction monitoring (MRM) was used to monitor precursor→product ion transitions at *m/z* 443.1→316.1 for RAL and *m/z* 446.1→319.0 for IS. The nebulizer gas, heater gas, ion spray voltage, heater temperature, curtain gas nitrogen and collisional activation dissociation were optimized at 50 psig, 60 psig, −4500 V, 300 °C, 35 psig and 7 psig, respectively. The compound-dependent parameters like declustering potential, entrance potential, collision energy and collision cell exit potential were maintained at −40.0 V, −10.0 V, −27.0 eV and −7.0 V, respectively. A dwell time of 100 ms was set for both the compounds. Data collection, peak integration, and calculations were performed using Analyst software version 1.4.2.

2.3. Preparation of standard solutions and quality control samples

The standard stock solution of RAL (1000 µg/mL) was prepared by dissolving its accurately weighed amount in methanol. Its working solution was prepared by appropriate dilution of stock solution in methanol:water (50:50, v/v). Calibration standards (CSs) and quality control (QC) samples were prepared by spiking blank plasma with standard spiking solutions. CSs were prepared at 2.0, 4.0, 12.0, 45.0, 90, 180, 480, 1200, 3000 and 6000 ng/mL concentrations while QC samples were prepared at 5000 ng/mL (HQC, high quality control), 2500 ng/mL (MQC, medium quality control), 6.0 ng/mL (LQC, low quality control) and 2.0 ng/mL (LLOQ QC, lower limit of quantification quality control) concentrations. Stock solution (1000 µg/mL) of the IS was prepared by dissolving 2.0 mg of RAL-d3 in 2.0 mL of methanol. An aliquot of 10 µL of this solution was further diluted to 25.0 mL in the same diluent to obtain a solution of 400 ng/mL. Standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at −70 °C until use.

2.4. Sample preparation

Prior to analysis, all frozen subject samples, CSs and QC samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked plasma/subject sample, 50 µL of IS was added and vortexed for 20 s. Thereafter, 50 µL of 0.1% formic acid in water was added and vortexed for another 20 s. Extraction of samples was carried out with 2.5 mL of DCM and *n*-hexane (50:50, v/v) on a rotospin for 10 min at 32*g*. The samples were then centrifuged at 3204*g* for 5 min at 10 °C. After centrifugation, 2.0 mL of the supernatant organic layer was transferred to an evaporation tube. The supernatant was evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a