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A novel ultrasensitive LC–MS/MS assay for quantification of intracellular raltegravir in human cell extracts☆

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ABSTRACT

An assay using ultrahigh pressure liquid chromatography and mass spectrometry detection was developed and validated for measurement of the HIV integrase inhibitor raltegravir (MK-0518) in human cell extracts. The assay is designed to utilize 200 μl of 70% MeOH cell extract derived from human peripheral blood mononuclear cells or human tissue samples. The assay is linear over a range from 0.0023 to 9.2 ng ml^{-1}. The average %CV (SD/Mean)*100 and %deviation ((observed – target)/target)*100 were less than 20% at the lower limit of quantification and less than 15% over the range of the curve. This assay is an accurate and highly sensitive method for determining raltegravir concentrations in cellular extracts with a lower limit 40 to over 100-fold lower than other methods in the literature. We also present a new processing method where a rapid spin through oil produced a significant increase in apparent intracellular raltegravir concentration compared with conventional processing.

1. Introduction

Raltegravir (RAL, MK-0518, Isentress®) is the first integrase inhibitor approved by the FDA for the treatment of adults and children older than two years with human immunodeficiency virus (HIV)-1 infection [1,2]. Integrase inhibitors exert their effects by preventing the insertion of viral DNA into the genome of the cell thereby suppressing viral replication [1,2]. Clinically, raltegravir is well tolerated, and has been shown to have a potent anti-HIV effect. A trial in antiretroviral-naïve adults compared raltegravir with efavirenz, both given in combination with tenofovir disoproxil fumarate and emtricitabine. At week 48 of therapy, 86.1% of the raltegravir group compared with 81.9% of the efavirenz achieved the primary endpoint of <50 copies ml^{-1} of HIV-RNA in plasma [3]. At week 156, the rates of viral suppression were 75.4% among those who received raltegravir versus 68.1% for efavirenz [4]. Raltegravir recipients experienced significantly fewer drug-related, adverse events than did efavirenz recipients. Interestingly, the time to suppression of plasma viremia was significantly shorter with raltegravir than efavirenz.

The pharmacokinetic characteristics of raltegravir are notable for high degrees of intra- and interpatient variability. In HIV-infected persons receiving the recommended dose of 400 mg orally twice daily in the fasting state, the area under the plasma concentration–time curve (AUC) ranged from 1685 to 12,770 ng h ml^{-1} at steady-state [5]. The highly variable nature of raltegravir's plasma concentrations is inconsistent with the favorable clinical response profile. There may be several mechanisms that complicate the pharmacokinetic/pharmacodynamic or exposure–response profile for this drug. It has been suggested that since raltegravir can be glucuronidated, secondary recycling of raltegravir by the enterohepatic system may occur resulting in a secondary spike in plasma raltegravir concentration and contribute to the variability in plasma concentrations [6]. The residence time of raltegravir on the preintegration complex is longer than the half-life of the free preintegration complex in the cell resulting in functionally irreversible inhibition, which is another contributing factor to the difficulty in finding a predictive pharmacokinetic parameter for efficacy from plasma data [7,8]. Finally, raltegravir exerts its inhibitory action on the integrase enzyme intracellularly; therefore, intracellular concentrations of raltegravir are likely a more accurate metric of virus exposure to the drug and response-related concentrations. To date there have been limited studies of intracellular raltegravir concentrations in peripheral blood mononuclear cells (PBMCs) and quite variable results with ranges of intracellular concentrations from 0% to approximately 10% of corresponding plasma concentrations [6,8]. Factors that may contribute to this variability include cell counting and processing.

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This paper describes our development and validation of an ultrasensitive LC–MS/MS based assay to quantify intracellular raltegravir concentrations. Additionally, we compared two different methodologies of cell processing: one typically used for the production of intracellular extracts and an alternate, more rapid procedure involving centrifuging the cells through an oil layer. The raltegravir MS assay was developed to measure minute quantities of raltegravir initially because expected intracellular concentrations were uncertain, and because this assay is intended to support pharmacokinetic studies of intracellular concentrations in PBMCs, the primary target cells for HIV, and in other tissues such as the gut associated lymphoid tissue (GALT), which may serve as a reservoir for the virus. In the case of GALT samples, the number of cells that can be reasonably be obtained by colonoscopy from human volunteers is very low, which increases the needed level of assay sensitivity.

2. Materials and methods

2.1. Chemicals and cells

Raltegravir (RAL) and C-13 labeled raltegravir (RAL-IS) were obtained from the manufacturer, Merck & Co. Inc. (Rahway, NJ). Acetonitrile (Optima grade), methanol (Optima grade), formic acid (99+% pure), glacial acetic acid (ACS grade) and ammonium acetate (ACS grade) were all obtained from Thermo Fisher Scientific (Fairlawn, NJ). Type I water was produced in the laboratory using a Millipore MilliQ Integral 3 system. Nyosil M25 oil (Nye Lubricants Inc., Fairhaven, MA) was obtained from TAI lubricants Hockessin, DE. Human PBMC's were either isolated from normal donors using elutriation or from patient samples using heparinized cell preparation tubes (Becton Dickinson catalog number 362753, Franklin Lakes, NJ).

2.2. Instruments

The chromatographic system consisted of two Shimadzu Prominence LC-20ADXR pumps, a Prominence SIL20ACXR autosampler, a Prominence CBM-20A controller, and a CTO20AC column oven connected to an API 5000 Mass Spectrometer (Applied Biosystem/Sciex, Foster City, CA) using an ESI interface. The data were acquired and analyzed using Analyst 1.5 software. Pure gases were produced by a Parker Balston SF5500 Gas Generator system (Haverhill, MA). Cells were counted using a Countess cell counter (Invitrogen, Life Technologies Corp., Grand Island, NY).

2.3. Standard curve stock preparation

A 1 mg ml^{-1} standard stock of raltegravir was prepared in 50% ACN:H_2O from an analytic weighing of raltegravir. This was serially diluted to give a 100 ng ml^{-1} stock working standard, which was further diluted to make 10× working standards. When corrected for salt content and purity the actual concentrations were of 0.023, 0.046, 0.092, 0.46, 0.92, 4.6, 9.2, 46, and 92 ng ml^{-1}. These were used to make the standard curve by adding 20 μl of these stocks to 180 μl of 70% MeOH. The actual standard curve final concentration ranges from 0.0023 to 9.2 ng ml^{-1}. A later confirmatory study showed good reproducibility over the range from 0.0025 to 10 ng ml^{-1}.

2.4. Quality control and validation sample preparation

A separate weighing of raltegravir solid was used to make a second 1 mg ml^{-1} stock solution of raltegravir in 50% ACN:H_2O This solution was diluted to make a 10,000 ng ml^{-1} preparation stock which was diluted 1:100 to make a final working stock solution of 100 ng ml^{-1}. A 750 μl aliquot of this stock was transferred to a 10 ml class A volumetric flask and diluted to volume with PBMC extract at a concentration corresponding to 2 million cells per ml. When corrected for salt content and purity this produced a 6.9 ng ml^{-1} QC high (QC-H). The PBMC extract was produced by extracting PBMC with 70% MeOH on ice and removing the cellular debris by centrifugation. The QC-H was diluted with more cell extract to produce concentrations of 0.069 ng ml^{-1} (QC-medium, QC-M), and 0.0069 ng ml^{-1} (QC-low, QC-L). Validation samples (VS) were prepared where 80 μl of a 1000 ng ml^{-1} stock were transferred to a 10 ml volumetric flask and brought to volume with PBMC cell extract. The 8 ng ml^{-1} validation sample was diluted 1:100 to produce a 0.08 ng ml^{-1} validation sample. The last two validation samples were produced by diluting volumes of 937.5 and 312.5 μl of the 0.08 ng ml^{-1} sample to 10 ml. This procedure produced final concentrations (after salt correction) of 7.4, 0.074, 0.0069, and 0.0023 ng ml^{-1} for the validation samples (VSH, 80% of high std; VSM (medium); and VSL, 3× the LLOQ) and the LLOQ (lower limit of quantitation).

2.5. Internal standard preparation

A working internal standard solution was prepared from reference standard by weighing approximately 1 mg of RAL-IS and dissolving with approximately 1 ml of 50% ACN:H_2O (the exact weight and volume will be the same). This was then diluted to make a 200 ng ml^{-1} stock solution which was stored at 4 °C. Aliquots were diluted daily with 50% ACN:H_2O to make a working internal standard concentration of 0.5 ng ml^{-1}.

2.6. Sample preparation

Standards were prepared in duplicate by adding 200 μl of water to labeled 1.5 ml snap top centrifuge tubes. This was followed by 180 μl of 70% MeOH, 20 μl of ammonium acetate at pH 4 and 20 μl of internal standard solution (except to the blank). Each of these components was added with an Eppendorf repeater pipette. Finally, 20 μl of standard solution was added to each tube except the blank and blank + IS tubes. The tubes were briefly mixed on a vortex mixer and pulse centrifuged to remove any reagent stuck to the side or top of the tubes.

Unknown and QC (three levels L, M, H in duplicate) samples were prepared by adding 200 μl of water, 20 μl of ammonium acetate at pH 4 and 20 μl of internal standard to appropriately labeled 1.5 ml snap top centrifuge tubes using an Eppendorf repeater pipette. 200 μl of the unknown or QC sample was then pipetted into its respective tube and briefly mixed on a vortex mixer and pulse centrifuged to remove any reagent stuck to the side or top of the tubes.

2.7. Chromatography and detector conditions

Raltegravir was separated with a Shimadzu Shim-pack XR-ODSII 2.0 mm × 75 mm column. The separation was run at a column temperature of 50 °C and samples were maintained at 15 °C. The overall run time was 8 min at a flow rate of 0.6 ml min^{-1}. Mobile phase A consisted of 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The gradient was a starting mixture of 25% B maintained for 1 min followed by a linear gradient from 25 to 90% B over 4 min, followed by a 1 min 100% B wash and a 2 min re-equilibration step. MS detection was performed with an API 5000 in MRM Positive mode, ESI. The Q1/Q3 was 445.2/361.2 for raltegravir and 451.1/367.2 for the internal standard. Other settings included and 80 ms dwell time, DP of 121, CE of 23, CXP of 50