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# Bioanalysis of Raltegravir, an Integrase Inhibitor in Human Plasma by Novel SPE–ESI–LC–MS/MS method and its pharmacokinetic application

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## ABSTRACT

This paper describes a simple, rapid and sensitive bioanalytical method based on liquid chromatography / tandem mass spectrometry (LC–MS/MS) for the determination of integrase inhibitor raltegravir in human plasma samples. Carbamazepine was used as an internal standard (IS). Analyte and the internal standard were extracted from 200 µL of human plasma *via* solid phase extraction. The chromatographic separation was achieved on a $C_{18}$ column by using a mixture of acetonitrile and 0.1% formic acid (90:10, v/v) as the mobile phase at a flow rate of 0.8 mL/min. The calibration curve obtained was linear ($r^2 \geq 0.99$) over the concentration range of 20.1–4007 ng/mL. Method validation was performed as per FDA guidelines and the results met the acceptance criteria. A run time of 2.0 min for each sample made it possible to analyze more number of samples in short time, thus increasing the productivity. The proposed method was found to be applicable to pharmacokinetic studies in humans.

**Keywords:** Raltegravir, human plasma, solid–phase extraction, LC–MS/MS, Pharmacokinetics

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## INTRODUCTION

Treatment of HIV is major medical concern in the world. However the complete eradication is not possible, hence the aim of the antiretroviral (ARV) treatment is minimizing the HIV viral load as low as possible in body. The availability of HAART (Highly active antiretroviral therapy) has improved success rate in the treatment of HIV infection by reducing the HIV related morbidity and mortality [1]. But even HAART is not very flourishing due to poor adherence to medication. Moreover, extensive use of anti–retroviral has led to development of drug resistance. There could be more chances of developing drug resistance from same class of drugs because of having the similar kind skeleton structure. Hence, there is a requirement for the new class of drugs which act by different mechanism lead to least prone for resistance and could be a compliment to the current treatment options.

Raltegravir is antiretroviral drug belongs to the new class of ARV called integrase inhibitor. This class of drugs prevents the replication of the virus by inhibiting the insertion of viral DNA into genome of the cell [2]. Raltegravir shown more effectiveness in reducing the viral RNA load in treatment of naïve HIV and patients with multi drug–resistant strains of the virus[3,4]. As per literature, numerous analytical methods have been reported in literature for determination of raltegravir levels in plasma which include HPLC–UV [5–8], HPLC–Fluorescence detection [9] and LC–MS/MS [10–15]. The LC–MS/MS estimation had the advantages over other techniques in–terms of selectivity and sensitivity. Hence, authors attempted to develop a suitable LC–MS/MS method for the determination of raltergravir in human plasma. Of all the above reported methods, only four methods [11–14] are comparable with the present work. The method proposed by Merschman *et al.* [11] and Takahashi *et al.* [12] utilizes multi step liquid–liquid extraction (extraction, separation, evaporation and reconstitution) with a longer chromatographic run time (> 7 min) which may not be favorable for routine drug analysis for pharmacokinetic/bioequivalence studies. Another method proposed by Long *et al.* [13] and Fayet *et al.* [14] utilizes a poor sample cleanup procedures like protein precipitation and involving complexity like gradient elution, typical mobile phase and longer chromatographic run time (> 10 min). Protein precipitation most likely to cause ion suppression as it fails to sufficiently remove endogenous compounds like lipids, phospholipids, fatty acids etc. But, today there is a more urge for the antiretrovirals in world and its always welcoming the good quality and high throughput techniques which significantly reduce the sample turnaround time with a promising quality in terms of consistency. The present proposed method significantly reduces the sample turnaround time with consistent

quality as it has employed the solid phase extraction with a direct injection and shorted chromatographic run time (2 min).

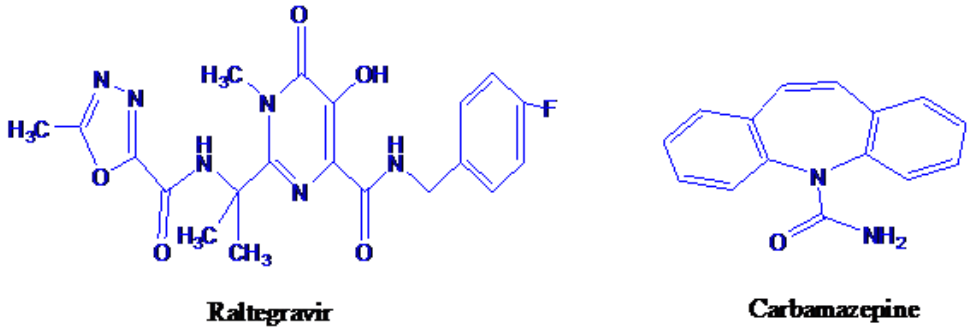


**Figure 1. Chemical structures of raltegravir and carbamazepine (IS).**

The present work describes a simple, selective and sensitive method, which employs solid phase extraction technique (SPE) for sample preparation and liquid chromatography with electrospray ionization – tandem mass spectrometry for quantitation of raltegravir in 200 µL of human plasma. SPE is the most popular sample clean up technique with its added advantages like good and consistent recoveries, minimal use of organic solvents (vs LLE) and greater possibility of automation. The method is validated as per the current regulatory guidelines and applicability of this method to the clinical pharmacokinetic study in healthy human volunteers also has been evaluated.

## MATERIALS AND METHODS

### Chemicals and materials

The reference standards of raltegravir and carbamazepine were obtained from Aurobindo pharmaceuticals Ltd, Hyderabad, India. Chemical structures are presented in the Fig. 1. Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile and methanol (HPLC grade) were purchased from J.T. Baker (Phillipsburg, USA). Extra pure formic acid was purchased from Fluka (Stinheim, Germany). Oasis HLB cartridges were obtained from Waters (Massachusetts, U.S.A). The control human plasma was obtained from Cauvery Diagnostics and Blood Bank (Secundrabad, India).

### Instrumentation and chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Supelco $C_{18}$ 50 × 4.6 mm 3.5 µm, a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) were used for the study. Aliquots of the processed samples (20 µL) were injected into the column, which was kept at ambient temperature. An isocratic mobile phase consisting of a

mixture of acetonitrile and 0.1% formic acid (90:10, v/v) was used to separate the analyte from the endogenous components and delivered at a flow rate of 0.8 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode using an MDS Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 ºC. The ion spray voltage was set at 5000 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 35, 35, 15 and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were 75, 27 and 11 for raltegravir and 75, 27 and 4 V for carbamazepine. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of m/z 445.3→361.2 for raltergravir and the m/z 237.1→194.1 for carbamazepine. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

**Preparation of plasma standards and quality controls**

Stock solutions of raltegravir and carbamazepine were prepared in methanol at a concentration of 1 mg/mL. From these stock solutions, appropriate dilutions were made to produce working standard solutions using a 50:50, v/v mixture of methanol and water as a diluent. Calibration curve (CC) standard solutions of raltegravir in blank plasma were prepared by spiking with an appropriate volume of the working solutions, giving final concentrations of 20.1, 40.2, 80.3, 201, 401, 803, 1606, 2404, 3206 and 4007 ng/mL for raltegravir. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at four different concentration levels of 20.2 (LLOQ), 57.7 (LQC), 443.7 (MQC1), 2016.8 (MQC2) and 3507.5 (HQC) ng/mL in blank plasma. All the prepared plasma samples were stored at −70 °C.

**Sample processing**

A 200 µL aliquot of human plasma sample was mixed with 20 µL of the internal standard working solution (1000 ng/mL of carbamazepine). To this, 200 µL of 5% formic acid solution was added after vortex mixing for 10 s. The sample mixture was loaded onto Oasis HLB cartridge (30 mg/ mL) that was pre–conditioned with 1.0 mL of methanol followed by 1.0 mL water. The extraction cartridge was washed with 1.0 mL of water followed by 1.0 mL of 5% methanol in water. Raltegravir and IS were eluted with 1.0 mL of mobile phase. Aliquot of 20 µL of the extract was injected into the LC–MS/MS system.

**Method validation**

The validation of the above method was carried out as per US FDA guidelines [16]. The parameters

determined were selectivity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and one hemolyzed plasma. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was checked with six different lots of K2–EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total). For checking the linearity standard calibration curves containing at least 10 points (non–zero standards) were plotted (20.1–4007 ng/mL). In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. Intra–day precision and accuracy were determined by analyzing six replicates at five different QC levels. Inter–day precision and accuracy were determined by analyzing six replicates at five different QC levels of three different runs. Recoveries of raltegravir and carbamazepine were determined by comparing the peak area of extracted analyte standard with the peak area of non–extracted samples. Recovery of raltegravir was determined at a concentration of LQC, MQC1, MQC2 and HQC, whereas for IS was determined at concentration of 1000 ng/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.6 times of the uppermost calibration standard were diluted two– and four–fold with blank plasma. The diluted samples were processed and analyzed.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (10 h), processed samples stability (Auto–sampler stability for 48 h, wet extract stability for 24 h), freeze–thaw stability (4 cycles), long–term stability (50 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

**Pharmacokinetic study**

A pharmacokinetic study was performed in healthy male subjects ($n$ = 6).The Ethics Committee (Hyderabad Independent Ethics Committee, Hyderabad, India) approved the protocol ant the volunteers provided written informed consent. The subjects were fasted for about 9 h before administration of the drug formulation. Blood samples were collected after oral administration of

raltegravir (400 mg) at pre–dose and 0.25, 0.5, 0.75, 1, 1.33, 1.66, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 16, 24, 36 and 48 h, in K2- EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 °C till their use. Plasma concentration–time profile raltegravir was analyzed by non–compartmental method using WinNonlin Version 5.1.

## RESULTS AND DISCUSSION

### Method development

Mass parameters were tuned in both positive and negative ionization modes for the analyte and internal standard. Good response was found in positive ionization mode. Chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution, desired intensity of the signals of the analyte and short run time. The presence of a small amount of formic acid in the mobile phase improved the detection of the analyte. It was found that a mixture of acetonitrile and 0.1% formic acid (90:10, v/v) could achieve this purpose and was finally adopted as the mobile phase. Supelco $C_{18}$ (50 mm × 4.6 mm, 3.5 µm) column gave good peak shapes and response even at lowest concentration level. The mobile phase was operated at a flow rate of 0.8 mL/min. The retention time of raltegravir and the IS were low enough (0.80 and 0.60 min) allowing a small run time of 2 min. A simple solid–phase extraction (SPE) technique was employed for the sample preparation in this work and provides high recoveries of the drugs. At the initial stages of this work, several compounds were tried for finding out a suitable IS in this analysis and finally carbamazepine was found to be the best for the purpose.

### Selectivity and chromatography

The degree of interference by endogenous plasma constituents with the analyte and the IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Figure. 2, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug–free plasma at the retention time of the analyte.

### Sensitivity

The lowest limit of reliable quantification for the raltegravir was set at the concentration of the LLOQ (20.1 ng/mL). The precision and accuracy at LLOQ concentration were found to be 2.51% and 99.22%.

### Matrix effect

No significant matrix effect was observed in all the six batches of human plasma for the

raltegravir at low and high quality control concentrations. The precision at LQC and HQC concentrations were found to be 2.05% and 1.20%.

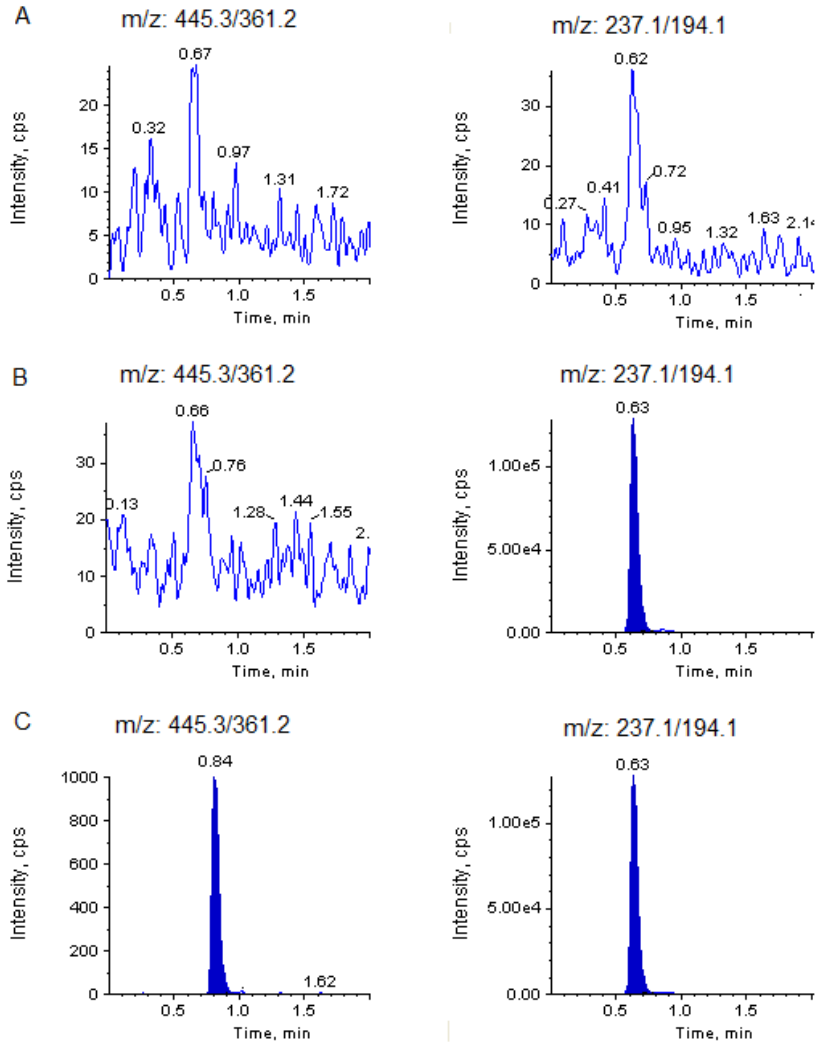


**Figure 2. Typical MRM chromatograms of raltegravir (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).**

**Linearity**

The ten–point calibration curve was found to be linear over the concentration range of 20.1–4007 ng/mL. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99.

**Precision and accuracy**

As shown in Table 1, the precision and accuracy of each analyte in the intra–day and inter–day

runs were within ± 15% at LQC, MQC1, MQC2 and HQC concentrations and within ± 20% at LLOQ QCs.

**Table 1. Precision and accuracy data for raltegravir in human plasma samples.**

| Analyte | Concentration added (ng/mL) | Intra–day precision and accuracy (*n*=12; 6 from each batch) | | | Inter–day precision and accuracy (*n*=18; 6 from each batch) | | |
|---|---|---|---|---|---|---|---|
| | | Conc. found (mean; ng/mL) | Precision (%) | Accuracy (%) | Conc. found (mean; ng/mL) | Precision (%) | Accuracy (%) |
| Raltegravir | 20.2 | 19.3 | 6.3 | 95.5 | 19.3 | 5.2 | 95.6 |
| | 57.7 | 54.4 | 2.3 | 94.5 | 54.3 | 2.7 | 94.1 |
| | 443.7 | 438.9 | 2.0 | 98.9 | 438.1 | 1.8 | 98.7 |
| | 2016.8 | 1969.9 | 1.8 | 97.7 | 1971.8 | 1.8 | 97.8 |
| | 3507.5 | 3475.6 | 1.9 | 99.1 | 3479.6 | 1.5 | 99.2 |

**Extraction efficiency**

Six replicates at low, medium–1, medium–2 and high quality control concentration were prepared for recovery determination. The recoveries of analyte and IS were good and reproducible. The mean overall recoveries (with the precision range) of raltegravir and IS were 101.86±6.09% and 113.14±4.61%, respectively.

**Dilution integrity**

The upper concentration limits can be extended to 6500 ng/mL and further diluted by 1/2 and 1/4 times with screened human blank plasma. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for 1/2 and 1/4 dilution samples were less than 2%.

**Table 2. Stability data for raltegravir in plasma samples (*n*=6).**

| Analytes | Stability test | QC (nominal conc.(ng/mL) | Mean ± SD (ng/mL) | % Change | Precision (% CV) |
|---|---|---|---|---|---|
| Raltegravir | Bench top[a] | 57.7 | 54.02 ± 1.4 | 6.4 | 2.6 |
| | | 3507.5 | 3438.2 ± 62.1 | 1.9 | 1.8 |
| | Auto sampler[b] | 57.7 | 54.1 ± 0.8 | 6.2 | 1.2 |
| | | 3507.5 | 3477.5 ± 37.1 | 0.9 | 1.1 |
| | Wet extract[c] | 57.7 | 54.0 ± 1.2 | 6.4 | 2.3 |
| | | 3507.5 | 3457.6 ± 26.5 | 1.4 | 0.8 |
| | FT[d] | 57.7 | 54.8 ± 1.6 | 5.0 | 2.9 |
| | | 3507.5 | 3470.1 ± 55.1 | 1.1 | 1.6 |
| | Long–term[e] | 57.7 | 54.6 ± 1.7 | 5.4 | 3.0 |
| | | 3507.5 | 3465.9 ± 49.6 | 1.2 | 1.4 |

[a] after 10 h at room temperature; [b] after 48 h in auto sampler; [c] after 24 h at 2–8°C; [d] after four freeze and thaw cycles; [e] at –70°C for 50 days

**Stability studies**

In the different stability experiments carried out viz. bench top stability (10 h), auto–sampler stability (48 h), repeated freeze–thaw cycles (4 cycles), wet extract stability (24 h at 2–8 °C) and

long term stability at –70 °C for 50 days, the mean % nominal values of the analytes were found to be within ±15% of the predicted concentrations for the analytes at their LQC and HQC levels (Table 2). Thus, the results were found to be within the acceptable limits during the entire validation.

**Pharmacokinetic study application**

In order to verify the sensitivity and selectivity of this method in a real–time situation, the present method was used to test for raltegravir concentrations in human plasma samples collected from healthy male volunteers (n = 6). The representative chromatograms of subject blank sample and a subject sample were shown in Figure 3. The mean plasma concentration vs time profile of raltegravir is shown in Figure. 4 and corresponding pharmacokinetic parameters are listed in Table 3.

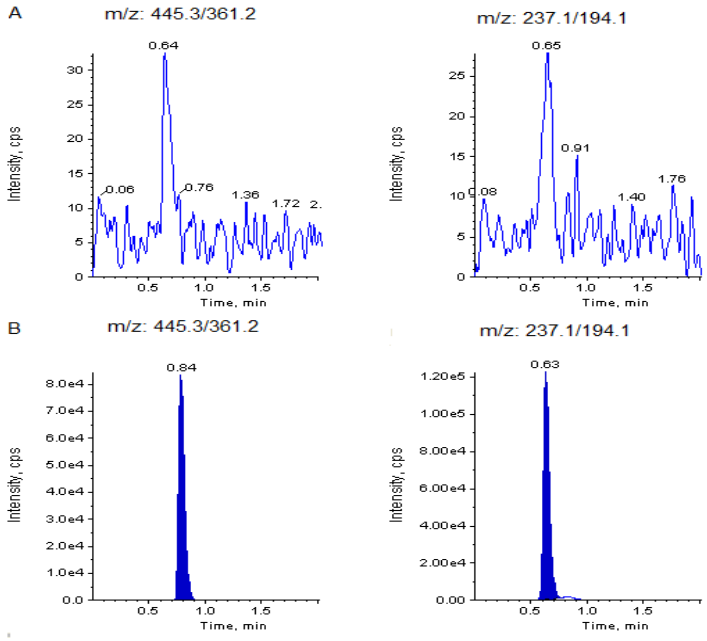


**Figure 3. MRM chromatograms resulting from the analysis of subject blank plasma sample (A) and a subject plasma sample (B), after the administration of a 400 mg oral single dose of raltegravir tablet.**

**Table 3. Pharmacokinetic parameters of raltegravir (n=6, Mean ± SD)**

| Parameter | Estimated value |
|---|---|
| $C_{max}$ (ng/mL) | 1119.528 ± 88.792 |
| $t_{max}$ (h) | 1.50 ± 0.18 |
| AUC0–t (ng h/mL) | 4252.006 ± 679.653 |
| AUC0–inf (ng h/mL) | 4471.482 ± 725.096 |
| t1/2 (h) | 10.658 ± 3.635 |
| Ke (h–1) | 0.0732 ± 0.02809 |
| Vd (mL) | 1377005.130 ± 386027.082 |
| CL (mL/h) | 92510.606 ± 21681.556 |

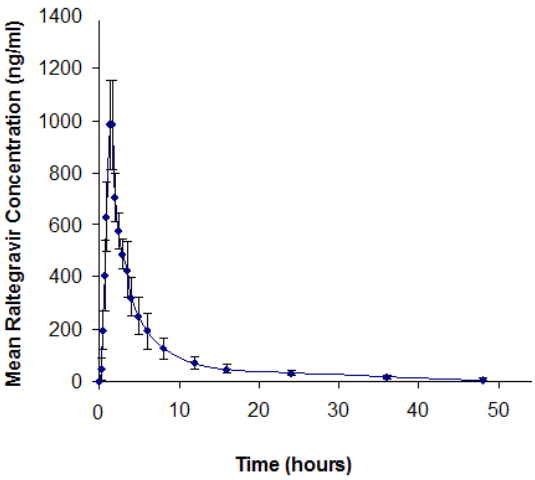


**Figure 4. Mean plasma concentration–time profile of raltegravir in human plasma following oral administration of raltegravir (400 mg tablet) to healthy volunteers (*n*=6).**

## CONCLUSIONS

The LC–MS/MS assay method described in this paper is rapid, simple, specific and sensitive for quantification of raltegravir in human plasma and is fully validated as per the FDA guidelines. The proposed method described the simple solid–phase extraction method with consistent and reproducible recoveries for the analytes from plasma. The method provided good linearity range which was successfully applied for pharmacokinetic study. The authors can still go for more sensitive LLOQ since, the reported LLOQ is giving pretty reasonable intense signal, but the purpose of the proposed method is for bioequivalence and 20.1 ng/mL is quite sufficient for the defined objective. A sample turnover rate of less than 2.0 min and simple extraction method makes it an attractive procedure in high–throughput bioanalysis of raltegravir.

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