SHORT NOTES

RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF RALTEGRAVIR IN HUMAN SALIVA

ABSTRACT

In this study, a high-performance liquid chromatographic method (HPLC) was developed, validated and applied for the determination of raltegravir in biological sample like saliva. Liquid- liquid extraction was performed for isolation of the drug and elimination of saliva interferences. Samples of saliva was extracted with 50µL of ortho phosphoric acid and 3mL of methanol was added and spiked with raltegravir. The chromatographic separation was performed on Agilent Eclipse C_{18} (100 mm × 4.6 mm, 3.5µm) column, by using 80:20 V/V acetonitrile: water as a mobile phase under isocratic conditions at a flow rate of 1.0 mL/min for UV detection at 240 nm. Retention time of raltegravir was found to be 1.030 min. Linearity was found to be in the range of 25-1000 ng/mL with regression equation y = 13864x + 40495 and correlation coefficient 0.999. The low % RSD value indicates the method is accurate and precise. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.76 and 2.28 ng/mL, respectively. It can be concluded that this validated HPLC method is easy, precise, accurate, sensitive and selective for determination of raltegravir in saliva.

Keywords: Raltegravir, HPLC, saliva, method validation, extraction.

INTRODUCTION

Raltegravir is chemically, N-(4-Fluorobenzyl)-5-hydroxy-1-methyl-2-(2-{[(5-methyl-1,3,4-oxadiazol-2yl) carbonyl]amino2-propanyl)-6-oxo-1,6-dihydro-pyrimidine carboxamide structure are shown in Fig.1. Raltegravir is an antiretroviral drug used to treat HIV infection[1,2]. The first of a new class of HIV drugs, the integrase inhibitors. Raltegravir targets integrase, an HIV enzyme that integrates the viral genetic material into human chromosomes, a critical step in the pathogenesis of HIV. The drug is metabolized away via glucuronidation. Different doses of raltegravir oral tablets were 200, 400 and 600 mg. Chewable tablets are of 25 and 50mg. A thorough literature survey has revealed that UV spectroscopy, HPLC[5,11] and LC MS [12-15] method are used for raltegravir with combination of other drugs, for its estimation in bulk, pharmaceutical dosage forms and biological samples. Till date there is no report the estimation of raltegravir in saliva samples. The aim of present research work for the development and validation of a HPLC method. An attempt was made to develop a simple, accurate, precise and rapid method for the estimation of raltegravir in biological samples like human saliva.

EXPERIMENTAL

MATERIALS AND REAGENTS

Raltegravir was gift sample from Hetero drugs. Acetonitrile HPLC Grade was purchased from Merck Chemical Company. The 0.45 µm pump Nylon filter was obtained from Advanced Micro Devices (Ambala Cantt, India) & whatman no 5 filter paper was obtained from Modern Science lab, (Nashik, India). Glasswares used were Class A grade.

Sample Preparation

In order to investigate the effects of medium on calibration curve linearity and equation parameter, working standard solutions of raltegravir are prepared in acetonitrile and saliva matrices. Human saliva was obtained from healthy volunteers and stored at -20°C until analysis. Stock standard solution of raltegravir was prepared by dissolving 10mg in 10mL acetonitrile and stored at -20°C for 1 month and protected from light. Further dilutions were prepared, by diluting stock solution with mobile phase to achieve calibration concentrations (25-1000 mg/mL^{-1}).

Extraction Process:

Trial-1 To 0.2mL of saliva samples, 50µL of ortho phosphoric acid and 3mL of n-hexane was added. The sample was mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuging, the supernant layer was separated and made up to 10mL with mobile phase [acetonitrile : water (80:20) v/v] .

Trial-2 To 0.2mL of saliva samples, 50µL of ortho phosphoric acid and 3mL of methanol was added. The sample was mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuging,

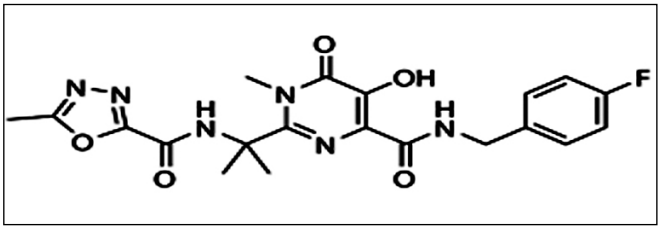

Fig.1: Structure of Raltegravir

supernant layer was separated and made up to 10mL with mobile phase [acetonitrile : water(80:20) V/V] .

Out of 2 trials performed, the 2nd trial was selected for further studies because when compared to other trials the 2nd trial facilitated good separation of saliva, with good peak symmetry.

Preparation of Sample Solution

To the extract solution, different concentrations of raltegravir was spiked to get the conc of 25-1000 mg/mL^{-1}

Validation of RP-HPLC Method

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantification, specificity and robustness were experimentally determined and the method was validated [16,17].

System Suitability Parameters

System suitability tests are an integral part of a chromatographic method. To ascertain its effectiveness, system suitability tests were carried out by injecting freshly prepared standard stock solution of 25mg/ml raltegravir in six replications and the parameters like retention time, peak area, plate number (N), and peak asymmetry of the samples were calculated.

Specificity

Specificity for an assay ensures that the signal measured comes from the substance of interest and there is no interference from excipient and/or degradation products and/or impurities. Specificity of the method was done by comparing the chromatogram of drug with the chromatogram of blank (mobile phase).

Linearity

Calibration and quality control samples were prepared by adding raltegravir solution in blank saliva. The amount corresponded to the saliva concentration of raltegravir which ranged from 25-1000 ng/mL^{-1}. The calibration curves for the saliva spiked by raltegravir were obtained by plotting raltegravir peak areas for the concentration range 25, 50, 75, 100,125, 250, 500 and 1000 ng/mL^{-1}.

Precision

Precision studies were carried out to ascertain the reproducibility of the proposed method.

Repeatability

Repeatability was determined by preparing six replicates of 125ng/mL, Raltegravir spiked with saliva. Equal volumes (20 µL) of each solution was injected separatly. The chromatograms were recorded and measure the peak response of drug was measured. The results were reported as %RSD.

INTERMEDIATE PRECISION

Intra Day Precision

Intraday precision study was carried out by preparing drug solution spiked with saliva of concentration (125ng/mL) and analyzing it at three different times in a day. The chromatograms were recorded and the peak response of raltegravir was measured.

Interday Precision

Interday precision study was carried out by preparing drug solution spiked with saliva of concentration (125ng/mL) and analyzing it at three different days to determine interday precision. The chromatograms were recorded and he peak response of raltegravir was measured. The results were reported as % RSD. The precision result showed a good reproducibility with percent relative standard deviation less than 2.

Accuracy

The accuracy of methods were evaluated by performing triplicate analyses of concentrations of 125ng/mL, 250 ng/mL and 500 ng/mL in saliva spiked drug samples.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$$LOD = 3.3 \times \sigma/S$$

Table I: Selection of mobile phase

Trials	Mobile phase	Observation	Remarks
1	Methanol: Water (50:50) V/V	Broad peak appearance	Not satisfactory
2	Acetonitrile : Water (50:50 V/V)	Negative Extra peak with tailing	Not satisfactory
3	Methanol : Acetatonitrile: water (50:40: 10 V/V/V)	Sharp peak with tailing	Not satisfactory
4	Methanol : Acetate buffer: water (50:40: 10 V/V/V)	Sharp peak with small fronting	Not satisfactory
5	Acetonitrile: Water (70:30 V/V)	Sharp peak with extra peak	Not satisfactory
6	Acetonitrile: Water (80:20 V/V)	Sharp peak appear	Satisfactory

$LOQ = 10 \times \sigma/S$

Where σ = the standard deviation of the response and S = slope of the calibration curve

Robustness

Robustness studies were carried by changing the flow rate of mobile phase from 0.8 to 1.2 mL/min, and wavelength from 238 to 242. Raltegravir samples made in triplicates and were analyzed.

Ruggedness

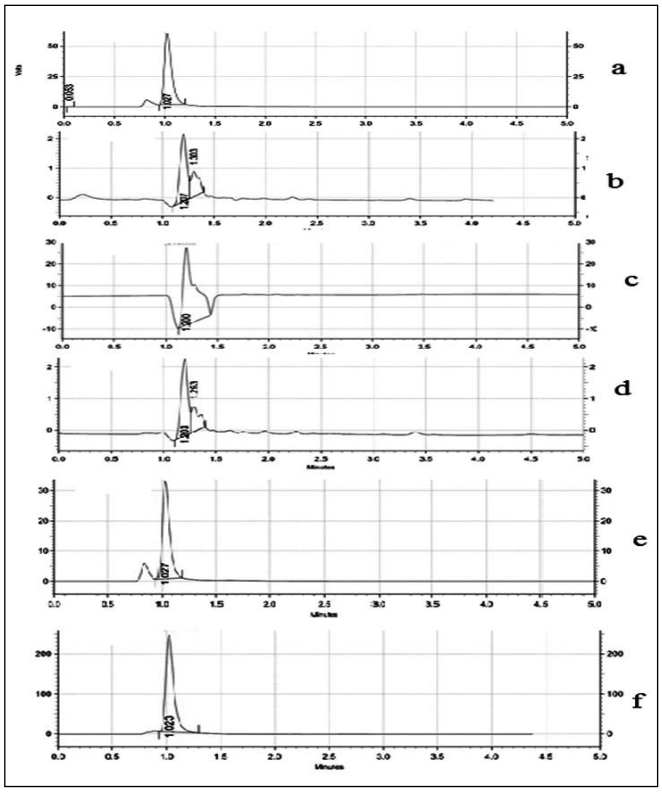

Fig. 2: Trails of different mobile phase a. Methanol : Water (50:50 V/V) b. Acetonitrile : Water (50:50 V/V) c. Methanol : Acetonitrile: water (50:40: 10 V/V/V) d. Methanol : Acetate buffer: water (50:40: 10 V/V/V) e.Acetonitrile: Water (70:30 V/V) f.Acetonitrile: Water (80:20 V/V)

Ruggedness studies were performed by preparing three replicates of 125 µg/mL of raltegravir and analysed by two different analysts and the results were reported as %RSD.

RESULTS

The Eclipse C_{18} (100 mm × 4.6 mm, 3.5µm) column was used and the mode of elution was isocratic. The flow rate 1.0m/min, injection volume was 20 µL and run time of sample was 5 min. Initially various mobile phase compositions were tried, to separate the ingredients. Out of 6 trials performed, the 6th trial the was selected because when compared to other trials, the 6th trial had the least in retention time, with good peak symmetry as mentioned in Table I and Fig. 2.

Extraction Process:

To 0.2mL of saliva samples, 50µL of ortho phosphoric acid and 3mL of methanol was added. The sample was mixed in a mechanical shaker for 20 min and centrifuged at 1000 rpm for 10 min. After centrifuging, the supernatant layer was separated and made up to 10ml with mobile phase [acetonitrile: water (80:20) V/V]. To the above solution, different concentrations of drug solution was spiked and the solution was injected.

HPLC Method Validation

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantification, specificity and robustness were experimentally determined and the method validated.

Specificity

Specificity of the method was done by comparing the chromatogram of drug (drug spiked with saliva) with the chromatogram of blank (saliva). The chromatogram of the blank was recorded and it did not show any peaks. The chromatogram of the drug is given in Fig. 3 and Fig.4 respectively.

System Suitability Parameters

System suitability tests are an integral part of a chromatographic method. They were used to verify that the reproducibility of the chromatographic system is adequate for the analysis. To ascertain its effectiveness, system suitability tests were carried out by injecting freshly prepared standard stock solution of raltegravir. Results are presented in Table II.

Table II. System Suitability Parameters

Parameters	Results
Retention time (min)	1.030
Theoretical plates	3882
HETP	0.053
Asymmetry	1.3

Table III. Repeatability studies of raltegravir

Concentration [ng/mL]	Area	Mean ±SD	%RSD
125	17590473	17571800± 122721.5	0.6984
125	17393653		
125	17472067		
125	17610121		
125	17621381		
125	17743102		

Linearity

Standard solutions of raltegravir in the concentration range of 25-1000ng/mL were injected into the chromatographic system and the peak areas were measured. A graph of peak areas (on Y-axis) versus concentration (on X-axis) was plotted and calibration graph was shown in Fig. 4. The regression equation was found to be y = 13864x + 40495. Correlation co-efficient was found to be 0.999.

Precision

Precision results were reported as %RSD. The precision results showed good reproducibility with percent relative standard deviation less than 2. Corresponding results are presented in Table III and IV.

Table V Accuracy studies of raltegravir

Saliva spiked Drug Conc (ng/mL)	Amount recovered (µg/mL)	% Recovery	% Mean recovery ±SD	%RSD
125	124.5	99.6	98.3±0.305	0.305
125	123.2	98.4		
125	21.2	97.2		
250	248.0	99.2	98.4±0.208	0.208
250	245.3	98.2		
250	244.6	97.8		
500	498.7	99.7	99.1±0.472	0.472
500	496.7	99.3		
500	491.5	98.3		

Accuracy

The accuracy of HPLC analysis tested by the recovery of raltegravir in saliva is summarized in Table V.

Robustness

Robustness studies were carried out by changing the flow rate of the mobile phase from 0.8 to 1.2 mL/min and by changing the wavelength from 238 to 242 nm. 125 ng/ml raltegravir was analyzed and the %RSD was determined and mentioned in Table VI.

Ruggedness

Ruggedness studies were performed by preparing three replicates of 125 ng/mL of raltegravir, analysing

Table IV: Precision of raltegravir

Concentration [ng/mL]	Intra Day Precision			Interday Precision		
	Area	Mean ±SD	%RSD	Area	Mean± SD	%RSD
125	17556190	18300506± 610644.8	0.033	17590473	17571800 ± 190919.2	1.09
125	17919377			17293653		
125	1785584			17172067		
125	18971789			17410121		
125	18512350			17621381		
125	18987487			17543102		

by two different analyst and the results are reported as %RSD and mentioned in Table VII.

Limit of Detection and Limit Of Quantification

The parameters LOD and LOQ were determined on the basis of response and slope of the regression equation. The LOD of the method was estimated to be 0.76 ng/mL and the LOQ of the method was estimated to be 2.28 ng/mL.

Table VI: Robustness studies of raltegravir

Factor	Level	RT	Mean AUC	Standed deviation	% RSD
Flow rate	0.8 (mL)	1.273	26692772	257157	0.96
	1.0 (mL)	1.027	21677414	165853.6	0.76
	1.2 (mL)	0.853	10709442	3586.7	0.03
Wave Length	238nm	1.027	10454212	180498.7	1.72
	240nm	1.023	19004709	167252.8	0.88
	242nm	1.020	18156644	284679	1.56

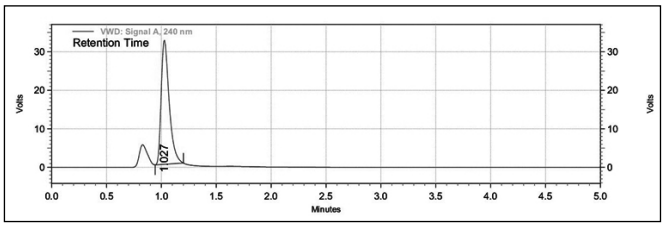

Fig. 3: Chromatogram raltegravir spiked with saliva

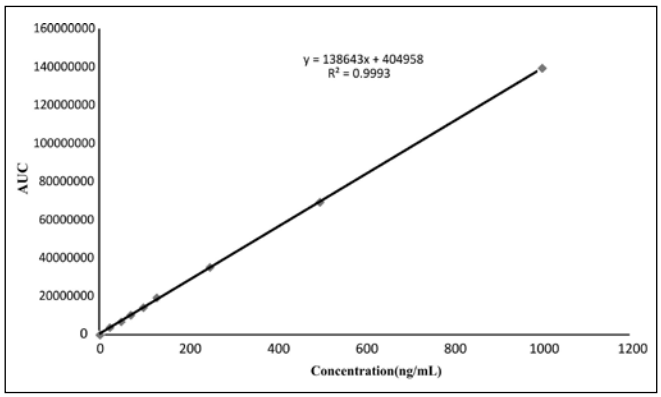

Fig. 4: Calibration curve of raltegravir spiked the saliva

Table VIII: Optimization of Chromatographic Conditions

Instrument	Agilent HPLC with UV detector
Mobile phase	Acetonitrile : Water (80:20 V/V)
Column	Eclipse C_{18} (100 mm × 4.6 mm, 3.5µm)
Flow rate	1 mL/min
Column temperature	Room temperature (20-25^{0}C)
Sample temperature	Room temperature (20-25^{0}C)
Wavelength	240nm
Injection volume	20µL
Run time	5 min
Retention time	1.030 min

CONCLUSION

A simple, rapid precise, accurate and robust RP-HPLC-UV method was developed, validated and applied for the determination of raltegravir in saliva samples and the optimized parameters are presented in the Table VIII. No interference from any components of the pharmaceutical dosage form was observed and the method has been successfully used to perform long-term and accelerate stability studies of raltegravir formulations.

ACKNOWLEDGEMENTS

The authors are grateful to management and principal Malla Reddy College of Pharmacy for providing necessary research facilities to carry out the research work and Hetero Labs, Hyderabad for providing the gift sample of the drug.

Table VII: Ruggedness of raltegravir

Concentration [µg/mL]	Analyst 1			Analyst 2		
	Area	Mean± SD	%RSD	Area	Mean ±SD	%RSD
125	17990476	17705124± 179491.7	1.01	17590473	17455134± 191430.2	1.09
125	17793653			17393652		
125	17472007			17472069		
125	17610125			17210126		
125	17621383			17321382		
125	17743102			17743102		

REFERENCE

1. Savarino A: A historical sketch of the discovery and development of HIV 1 integrase inhibitor, **Expert Opin Investig Drugs**, 2006, 15(12) 1507-1522.
2. Cocohoba J., Dong B. J., Raltegravir.: The first HIV integrase inhibitor, **Clin Ther**, 2008, 30(10) 1747-65.
3. Siddartha B. and Sudheer Babu I.: UV – spectrophotometric method for estimation of raltegravir in bulk and tablet dosage form, **Int J. Pharma**, **Chemical and Bio Sci,** 2014, 4(4) 807-811.
4. Girija B. Bhavar, Sanjay S., et al.: Simple spectrophotometric method for estimation of raltegravir potassium in bulk and pharmaceutical formulations, **J. App Pharma Sci,** 2013, 147-150.
5. Jean Marie Poirier, Pascal Robidou, Patrice Jaillon.: Quantification of the HIV-integrase inhibitor Raltegravir in human plasma by high performance liquid chromatography with fluorescence reduction, **J. chromatogr B**, 2008, 867 277-281.
6. Jasmine A., Talameh, et al.: Quantifying the HIV-1 integrase inhibitor raltegravir in female genital tract secretions using high performance liquid chromatography with ultraviolet detection, **J. Chromatogr B**, 2010, 878 92-96.
7. Lakshmi T., Annapurna A. and Krishna R Gupta.: HPLC method development and validation for determination of raltegravir in blood plasma, **Int J. Pharm Bio Sci,** 2015, 6(1) 113 – 120.
8. Notari S., Tommasi C., Nicastri E., et al.: Simultaneous determination of maraviroc and raltegravir in human plasma by HPLC-UV method, **Inter Union Biochem Mol Biology life,** 2009**,** 61(4) 470-475.
9. Poirier J.M., Robidou P. and Jaillon P.: Quantification of the HIV integrase inhibitor raltegravir (MK-0518) in human plasma by high performance liquid chromatography with fluorescence detection, **J. Chromatogr B Analyt Technol Biomed Life** Sci, 2008, 67(2) 277-81.
10. Talameh J.A., Rezk N.L. and Kashuba A.D.: Quantifying the HIV-1 integrase inhibitor raltegravir in female genital tract secretions using high performance liquid chromatography with ultraviolet detection, **J. Chromatogr B Analyt Technol Biomed Life Sci,** 2009, 878(1) 92-6.
11. Takahashi M., Konishi M., et al.: A conventional LC-MS method developed for the determination of plasma raltegravir concentrations, **Biol Pharm Bull**, 2008, 31(8) 1601-04.
12. Acosta E.P., Long M.C et al.: A sensitive HPLC-MS-MS method for the determination of raltegravir in human plasma, J. **Chromatogr B** ,2008, 867 165- 171.
13. Fayet A., Béguin B., Zanolar: LC– tandem MS assay for the simultaneous measurement of new antiretroviral agents: Raltegravir, maraviroc, darunavir, and etravirine, **J. Chromatography B**, 2009, 811(11-12) 1057-69.
14. Heine R.T., Hillebrand M.J., Rosing H, Gorp E.C.V., Mulder J.W., Beijnen J.H and Huitema AD.: Quantification of the HIV-intergrase inhibitor raltegravir and detection of its main metabolite in human plasma, dried blood spots and peripheral blood mononuclear cell lysate by means of high performance liquid chromatography tandem mass spectrometry, **J**. **Pharm Biomed Anal**, 2009, 49 451-458.
15. Wang L. Z., Lee L. S., Flexner C and Goh BC.: Simultaneous determination of raltegravir and raltegravir glucuronide in human plasma by liquid chromatography tandem mass spectrometric method, **J. Mass Spectrom**, 2011, 462 02-228.
16. Michael E., Schartz S., and Krull, Analytical Method Development and Validation, 2004, pp. 25-46.
17. Validation of analytical procedures: msethodology, ICH Harmonized Tripartite Guideline, **ICH Q2B,** May 1997, pp. 1-15.

[a] Malla Reddy College of Pharmacy,
(Affiliated to Osmania University)
Maissamma Guda , Secundrabad - 500 014, Telangana, India

Vijaya Sri K.[a]* Shiva Kumar M.[a], Madhuri M. A.[a] and K. Suresh[a]

***For Correspondence : E-mail: vijayasree_2002@yahoo.co.in**

(Received 15 July 2018) (Accepted 08 October 2018)

https://doi.org/10.53879/id.56.09.11506