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SIMULTANEOUS ESTIMATION AND VALIDATION OF RALTEGRAVIR, EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE IN PURE DRUG FORM BY UV-SPECTROPHOTOMETRY

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ABSTRACT

The main objective of present research work was to develop and validate the UV- spectrophotometric methods for the simultaneous estimation of Raltegravir, Emtricitabine, and Tenofovir Disoproxil Fumarate pure drug form method is based on simultaneous equation analysis by using Methanol as a solvent. The simultaneous equation method depends on mainly that among three components (Raltegravir,Emtricitabine and Tenofovir Disoproxil Fumarate) each of which absorbs at the λmax of each other, Results: The λmax of Raltegravir, Emtricitabine, and Tenofovir Disoproxil Fumarate was found to be 328nm, 241nm and 259nm respectively the linearity range of Raltegravir, Emtricitabine, Tenofovir Disoproxil Fumarate between 5-55,5-30 and 5-55µg/ml respectively with correlation coefficient 0.999, 0.998 , 0.998 respectively. The % RSD for intraday precision and interday precision of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate was found to be 0.43, 0.67, 0.49 and 0.17, 0.49, and 0.43. In both, the cases values were within the acceptance limit of less than 2%. The mean percent recovery for Raltegravir, Emtricitabine, Tenofovir Disoproxil Fumarate were found to be 100.3, 100.65, 100.3 respectively. The method was validated for various parameters as per ICH guidelines and the results are found to be in acceptable limits.

INTRODUCTION

HIV (Human Immunodeficiency Virus) is a retrovirus that gradually attacks the body's immune system, which protects human body against illness. HIV infected person becomes a easy target for opportunistic infections and diseases. This virus multiplies in T-helper cell which is the white blood cells (CD4) and gradually depletes them. The two main types are HIV-1 and HIV-2. HIV-1 is the most common type found worldwide. When HIV targets and invades these cells, it decreases the body's capacity to combat other diseases.HIV infected person would develop AIDS in 10 to 15 years which is last clinical Stage. HIV mainly found in blood, semen, vaginal and anal fluid and breast milk. However, it cannot be transmitted through sweat, saliva or urine [4]. Currently, there is no cure for HIV but with early diagnosis and effective Antiretroviral (ARV) treatment, people with HIV can live a long and normal, healthy life. Therefore, it is important to take correct treatment regularly. The drugs currently available for HIV block the replication by interfering at various stages of the life cycle. These drugs have their own toxicities and many have reported development of resistance. Treatment of HIV started as monotherapy initially, and then multiple drugs in regimens were given

where patients had to consume 11-16 tablets per day. Toxicity, resistance and adherence still remain crucial issue. We need long acting depot preparations which would be efficacious for prevention, treatment and have fewer side effects. To implement test and treat policy promoted by WHO, regular supply of cost effective antiretroviral drugs and newer drugs which would get approved remains a challenge for developing countries[4]. A new drug combination regimen, consisting of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate (TDF), used for treatment of HIV-1 infection. This new drug combination regimen is applicable to the treatment of drug-naive HIV-1-infected patients. This opens new perspectives for the design of multiple drug combination regimens targeting different enzymes involved in HIV-1 replication, i.e. integrase and reverse transcriptase. These multiple (triple or quadruple) drug combinations should be aimed at once daily dosing

Raltegravir has recently been proposed as part of a drug regimen (in combination with Emtricitabine and Tenofovir Disoproxil Fumarate)[5]. Raltegravir is the first-in-class of a new class of HIV-1 inhibitors targeted at the proviral DNA integration into the cell genome. Targeted at a new site in the HIV-1 replicative cycle, namely the HIV-1 integrase.Indicated for patients with resistance to other classes of drugs . It has proven to be safe and efficacious in treatment-experienced patients with multidrug-resistant virus. Raltegravir plus optimized background therapy provided better viral suppression[6].

Emtricitabine, a nucleoside reverse transcriptase inhibitor, is phosphorylated by cellular enzymes to Emtricitabine 5′-triphosphate which, in turn, inhibits the activity of HIV-1 (HIV) reverse transcriptase by competing with the endogenous substrate. Incorporation of the triphosphate into the viral DNA causes chain termination, thereby inhibiting viral replication.In adult patients infected with HIV, combination therapy including Emtricitabine 200mg once daily was as effective as triple therapy.In addition, 85% of Emtricitabine recipients maintained virological success during 96 weeks of therapy.These early clinical trials such that phase I/II studies carried out demonstrated a concordance between dosage regimens with respect to antiviral activity, safety and pharmacokinetics to support the dose selection strategy for the long-term therapeutic trials of Emtricitabine.Emtricitabine-based therapy was generally well tolerated, most adverse events being mild to moderate in intensity[7].

Tenofovir Disoproxil Fumarate (TDF) for first-line therapy in treatment-naive patients with HIV-1 infection[5]. Tenofovir, an adenosine nucleotide analog with potent activity against retroviruses use as Viread). Tenofovir's efficacy in suppressing viral replication, favorable safety profile, and long half-life made it an ideal choice as the first antiretroviral[9] with activity against retroviruses, including HIV-1, HIV-2 and hepadnaviruses[8].The use of antiretroviral medications in HIV-negative individuals as pre-exposure prophylaxis (PrEP) is a promising approach to prevent HIV infection. Tenofovir Disoproxil Fumarate (TDF) and emtricitabine exhibit desirable properties for PrEP including: favourable pharmacokinetics that support infrequent dosing,few major drug-drug or drug-food interactions,an excellent clinical safety record and pre-clinical evidence for efficacy. Several large, randomized, controlled clinical trials are evaluating the safety and efficacy of TDF and emtricitabine for this new indication**[10].**

Combining different drugs that differ in their mode or target of action may be beneficial from three viewpoints such as

- Synergistic activity translating into higher antiviral potency
- Reduced likelihood for the emergence of virus-drug resistance
- Lesser chance for toxic side effects due to lower dosage levels[4].

In combination studies evaluating the in Vitro antiviral activity of emtricitabine and tenofovir together, synergistic antiviral effects were observed[4].Literature survey

revealed that a few analytical methods have been reported for the determination of Raltegravir in pure drug and in pharmaceutical dosage forms using HPLC and LC-MS either in single or in combined forms.But no analytical method was found for the simultaneous estimation of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate (TDF), by UV spectroscopy[11-15]

RALTEGRAVIR:

Raltegravir is an integrase inhibitor, the first of the class of antiviral agents active against the human immunodeficiency virus (HIV) that targets the viral integrase.

Raltegravir inhibits HIV integrase to prevent the viral genome being incorporated into the human genome. Raltegravir is primarily metabolized by glucuronidation. Raltegravir is used in combination with other antiretroviral agents in the treatment of HIV infection[1].

Drug profile of Raltegravir

Chemical structure:

IUPAC Name: N-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-2-{2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl}-6-oxo-1,6-dihydropyrimidine-4-carboxamide

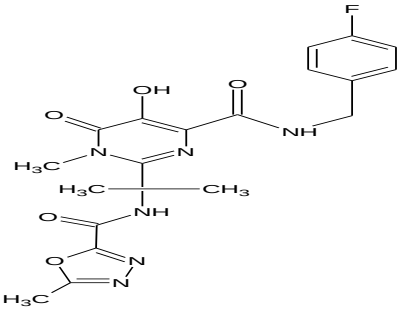
Molecular formula:$C_{20}H_{21}FN_6O_5$

Molecular weight :444.4 g/mol

Characteristics : White to off-white powder

Category : Anti-Retroviral Agents

Solubility: Solubleinwater,slightly soluble in methanol very slightly soluble ethanol and acetonitrile[1].



EMTRICITABINE

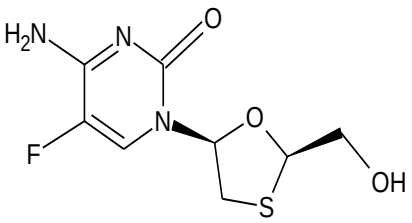
Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI)

Emtricitabine is a cytidine analogue.The drug works by inhibiting HIV reverse transcriptase, preventing transcription of HIV from RNA to DNA.

Used to treat the HIV infection in adults or combined with tenofovir alafenamide for the prevention of HIV-1 infection in high risk adolescents and adults[2].

Drug profile of Emtricitabine

Chemical structure:



IUPAC Name: **4**-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Molecular formula:$C_8H_{10}FN_3O_3S$

Molecular weight :247.25 g/mol

Characteristics : Solid, white to off white powder

Category : Anti-Retroviral Agents

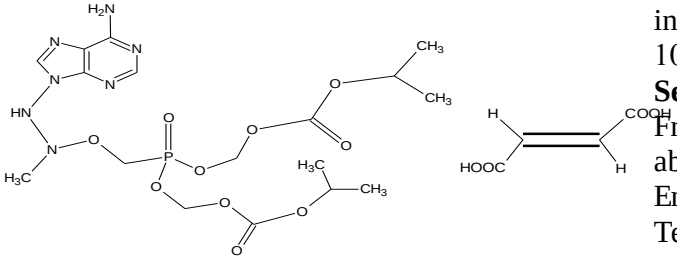
Solubility : Soluble in water , freely soluble in methanol[2].

TENOFOVIR DISOPROXIL FUMARATE :

Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analog reverse transcriptase inhibitors (NtRTIs), which block reverse transcriptase, an enzyme necessary for viral production in HIV-infected individuals. Tenofovir Disoproxil Fumarateis the fumarate salt of the prodrug *tenofovir disoproxil*. Tenofovir disoproxil is absorbed and converted to its active form, *tenofovir*, a nucleoside monophosphate (nucleotide) analog. Tenofovir is then converted to the active metabolite, *tenofovir diphosphate*, a chain terminator, by constitutively expressed enzymes in the cell. Tenofovir diphosphate inhibits HIV-1 reverse transcriptase and the Hepatitis B polymerase by direct binding competition with the natural deoxyribonucleotide substrate (deoxyadenosine 5'-triphosphate) and, after integration into DNA, causes viral DNA chain termination .Tenofovir Disoproxil Fumarateis prescribed to treat HIV and chronic hepatitis B virus (HBV) in adults[3].

Drug profile of Tenofovir Disoproxil Fumarate

Chemical structure :



IUPAC Name :(2E)-but-2-enedioic acid; bis({[(propan-2-yloxy)carbonyl]oxy}methyl) {[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methanephosphonate

Molecular formula :$C_{23}H_{34}N_5O_{14}P$

Molecular weight :635.5 g/mol

Characteristics : White, fine, powder-like crystals

Category : Anti-Retroviral Agents

Solubility : Soluble in methanol, slightly soluble in water[3].

Materials and Methods

Emtricitabine And Tenofovir Disoproxil Fumarate standards were obtained as gift sample from Mangalam Organics LTD Mumbai and Raltegravir from Hetero Drugs Limited, Hyderabad. All the chemicals were of HPLC grade and are obtained from the stores of Government College of Pharmacy, Bengaluru.

Instruments used

Electronicanalyticalbalance,Shimadzu-1800UV-Spectrophotometer,UltrasonicbathSonicator , a pair of 1cm matched quartz cells was used in this study.

Selection ofsolvents

By carrying out solubility profile study and literature survey, it was found that Raltegravir, Emtricitabine And Tenofovir Disoproxil Fumarate are soluble in methanol and water. Hence methanol were chosen for the UV-Spectrophotometer analysis of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate

Preparation of standards stock solutions

10 mg of each standard Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate were weighed separately, into three 10 ml volumetric flask and dissolve in methanol respectively to get the concentration of 1000µg/ml, further 1ml into 10ml Volumetric flask and dissolve in methanol to get concentration of 100µg/ml.

Selection of analyticalwavelength

From the abovestandardstocksolution,0.5ml of Emtricitabine and 1ml of Raltegravir and Tenofovir Disoproxil Fumarate wastakenseparatelyinto10ml volumetricflask and diluted with the solvents and these solutions were scanned in the UV region of 200-400nm. Maximum absorbance was seen in the wavelength of 241nm for Emtricitabine, 259nm for Tenofovir Disoproxil Fumarate and 328nm for Raltegravir. Hence all absorbance measurements were made at 241nmforEmtricitabine, 259nmforTenofovir Disoproxil Fumarate and 328nm for Raltegravir**Preparation of standard stock solution**

Stock I solution

Standard stock solution of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate were prepared separately by dissolving 10mg of Raltegravir, 10mg of Emtricitabine, 10mg of Tenofovir Disoproxil Fumarate in methanol and made the volume up to 100 ml with same solvent

Stock II solution

From the above stock solutions 1ml of the each aliquots were pipetted out ina 10ml volumetric flask separately and the volume was made up to the mark with same solvent to obtain the final concentration of 20µg/ml of Emtricitabine, 20µg/ml of Tenofovir Disoproxil Fumarate and 20µg/ml of Raltegravir

Selection of wavelength

Working standard solutions of 20µg/ml of Emtricitabine, 20µg/ml of Tenofovir Disoproxil Fumarateand 20µg/ml of Raltegravirwere further prepared by appropriate dilution of standard stock solutions. Overlay spectrum of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate were scanned and from which the wavelengths of 328nm, 241nm and 259nm were selected for Raltegravir, Emtricitabine

and Tenofovir Disoproxil Fumarate respectively for further studies.

Simultaneous Equation Method

Three wavelengths required in this method are selected at which one component shows maximum absorbance, while remaining shows considerable absorbance.

Considering this fact, wavelengths 328 nm, 241nm and 259nm (λmax of all three drugs) were selected for the estimation of Raltegravir, Emtricitabine, Tenofovir disoproxil fumarate by simultaneous equation method. At all selected wavelengths the absorbance and absorptivity values of three drugs were determined [16]. The absorbance of the mixture at 328 nm, 241 nm and 259nm may be expressed as follows:

A1 = ax1bCx + ay1bCy + az1bCz……at 328 nm

A2 = ax2bCx + ay2bCy + az2bCz……at 241 nm

A3 = ax3bCx + ay3bCy + az3bCz……at 259 nm

For measurements in 1cm cell b = 1. Hence

A1 = ax1Cx + ay1Cy + az1Cz

A2 = ax2Cx + ay2Cy + az2Cz

A3 = ax3Cx + ay3Cy + az3Cz

Where the absorbance of diluted sample solution at 328, 241 and 259 nm are A1, A2and A3 respectively, The absorptivity of Emtricitabine at 241nm ax1, ax2 and ax3 at 259 nm are ay1, ay2 and ay3 respectively, The absorptivity of Raltegravir at328nm are az1, az2 and az3 respectively, Cx, Cy and Cz are the concentration of Emtricitabine, Tenofovir andRaltegravirrespectively in the diluted samples. Using above equations 1, 2 and 3 the concentrations of component X (Raltegravir)component Y (Emtricitabine), and component Z (Tenofovir) in the sample mixture can be determined.

Application of the Proposed Method.

The powder equivalent to about 40 mg of Raltegravir ,20 mg of Emtricitabine and 30mg of Tenofovir was transferred to a 100ml volumetric flask. Approximately 75ml of diluent was added to the flask and mixed well by sonicating it for 15 min. Then flask was adjusted to volume with diluent. The solution was filtered using whatmann filter 5 paper intovolumetric flask. The resulting solution was used as a working sample solution which contain 200µg/ml of Emtricitabine, 300µg/ml of Tenofovir and 400µg/ml of Raltegravir0.1ml of filtrate was taken in a 10ml volumetric flask and made the volume with methanol solvent. The above solution was analyzed at 328, 241 and 259nmwavelengths and values of the absorbance were substituted in respective equations 1, 2 and 3 to obtain the concentration of Raltegravir,Emtricitabine and Tenofovir disoproxil fumarate .

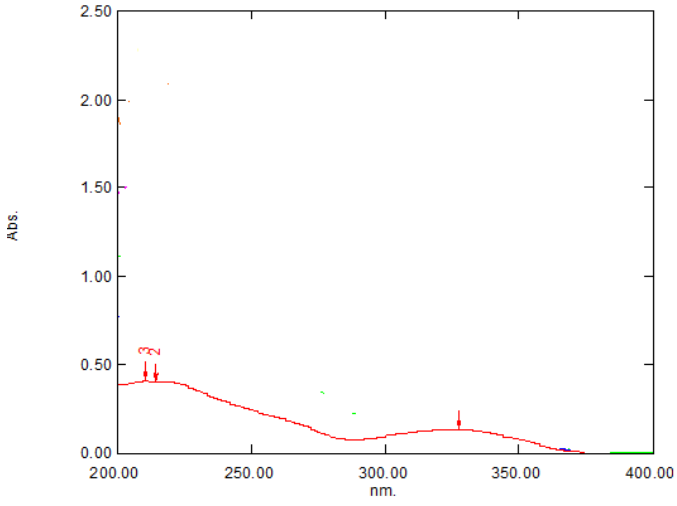


Fig 1 Raltegravir

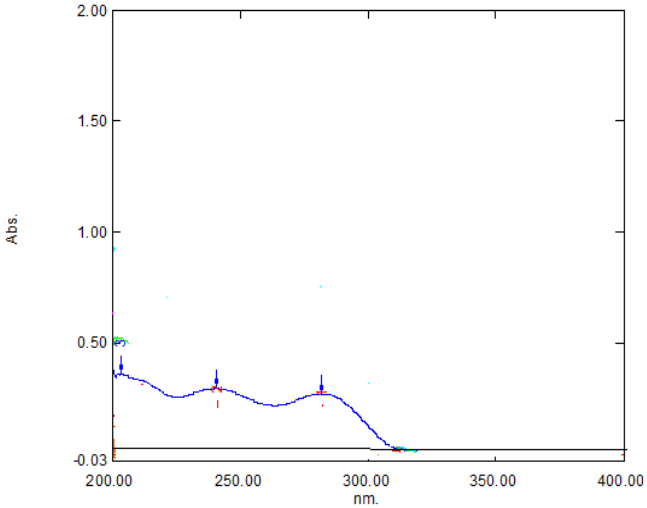


Fig 2 Emtricitabine

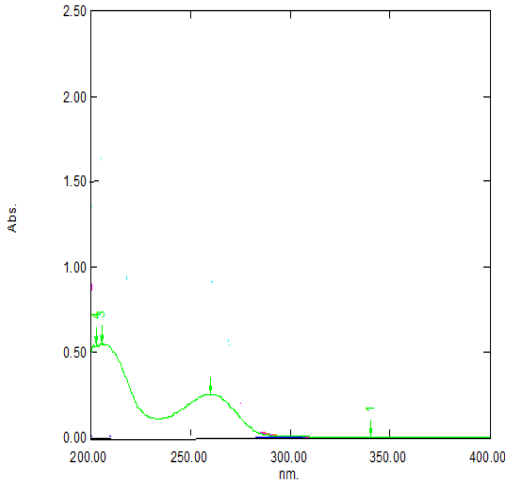


Fig 3 Tenofovir Disoproxil Fumarate

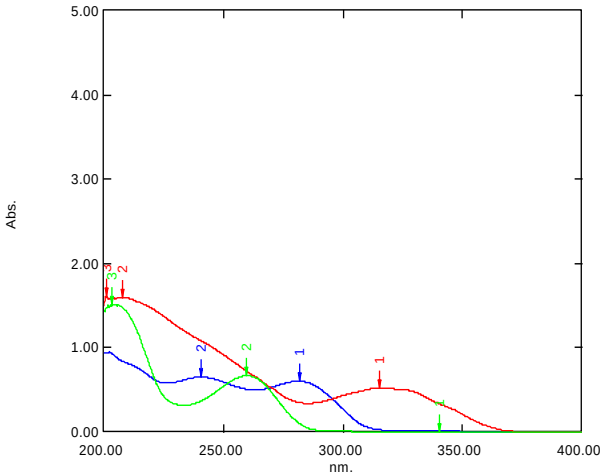


Fig 4 Mixture

Validation of developed method

Linearity

Linearity was evaluated by preparing the solutions having the concentration range of 5-55µg/ml of Raltegravir,5-30 µg/ml of Emtricitabine and 5-55 µg/ml of Tenofovir Disoproxil Fumarate.The calibration curves were obtained by plotting absorbance against concentration (µg/ml) for three different wavelengths (328nm, 241nm, 259nm). Standard deviation (SD), slope, intercept, and correlation coefficient of determinations (r2) of the calibration curves were calculated to ascertain the linearity of the method.

Method precision (repeatability)

The precision of the instrument was checked by repeated scanning and measurement of the absorbance of solution(n=6) for Emtricitabine (20µg/ml),Tenofovir Disoproxil Fumarate(20µg/ml) and Raltegravir (20µg/ml) without changing the parameter of the proposed UV method. The %RSD was calculated.

Intermediate Precision (reproducibility)

The intraday and interday precision of the proposed method was determined by analyzing the corresponding responses on the same day and next day for three different concentrations of standard solution of Emtricitabine (5,10,15µg/ml), Tenofovir Disoproxil Fumarate (10, 20, 30µg/ml) and Raltegravir(10, 20, 30µg /ml). The result was reported in terms of relative standard deviation (%RSD).

Accuracy

Accuracy of the proposed method was determined using recovery studies by spiking method. The recovery studies were carried out by adding different amounts (50, 100 and 150%) of the pure drug to the pre-analysed formulation. The solutions were prepared in triplicates and the % recovery was calculated.

Limit of Detection and Limit of Quantification

The limit of quantification (LOQ) and limit of detection (LOD) were based on the residual standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in International Conference on Harmonization guidelines Q2 (R1) LOD = 3.3 × σ/S, LOQ = 10 × σ/S Where σ = the standard deviation of the response and S = slope of the calibration curve.

Ruggedness Studies

Ruggedness studies were performed by preparing 6 replicates of 20µg/ml of Emtricitabine, 20µg/ml of Tenofovir Disoproxil Fumarate and 20µg/ml of Raltegravirand analyzing by two different analysts and on two different instruments and the results are reported as %RSD.

Results and Discussion

The method was validated according to ICH guidelines [15-17] in order to determine the linearity, precision, accuracy and ruggedness of the method. The summary of optical parameters is shown in Table No 1

Linearity

The absorbance of the solutions of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate was determined at three wavelengths i.e. 328nm, 241nm, 259nm and the calibration curves were plottedand the overlay spectra's of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate

Method precision (repeatability)

Repeatability was determined for all the drugs and % RSD was found to be less than 2 which has shown in Table No. 2

Intermediate Precision (reproducibility)

The precision of the developed method was expressed in terms of percent relative standard deviation (% RSD). These results show reproducibility of the assay. The % RSD values were found to be less than 2 that indicate this method precise for the determination of the pure form. The interday and intraday precision results were mentioned in Table No 3

Accuracy

Accuracy is determined by performing recovery studies at 3 levels in which known amount of analyte shall be added and recovery shall be carried out in three replicates of each concentration level and the % recovery was calculated. The accuracy results are shown in Table No 4

Ruggedness Studies

Ruggedness was performed by two different analysts and two different instruments and the results of the study were given in **Table 5** and % RSD obtained was less than 2 which is within the acceptance limitsRuggedness data of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate by analyst 1 and instrument 1 by UV method. Ruggedness results are shown in Table No 5

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. LOD & LOQ values are 0.508 and 1.54 for Emtricitabine, 0.569 and 1.72 for TenofovirDisoproxil Fumarate and 0.167 and 0.508 for Raltegravir

Table No.1: Linearity and range report of Raltegravir, Emtricitabine and Tenofovir Disoroxil Fumarate

Parameter	Raltegravir	Emtricitabine	Tenofovir Disoproxil Fumarate
Linearity Range Beer's law limit(µg/ml)	5-50µg/ml	5-30µg/ml	5-50µg/ml
Wavelength	328	241	259
Regression Equation (y=mx+c)	0.145x - 0.017	0.136x + 0.012	0.172x + 0.019
Slope(m)	0.145	0.136	0.172
Intercept(c)	-0.017	0.012	-0.019
Correlation Coefficient (r)	0.999	0.998	0.998
Molar Absorptivity	139.11	275.22	230.50
Precision	0.7077%	0.512%	0.598%
Limit of detection	0.167	0.508	0.569
Limit of quantification	0.508	1.54	1.724

Table No 2 Method Precision data of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate

Drugs	Raltegravir		Emtricitabine		Tenofovir Disoproxil Fumarate	
Sl.No	**Conc µg/ml**	**Abs at 328 nm**	**Conc µg/ml**	**Abs at 241nm**	**Concµg/ml**	**Abs at 259 nm**
1	10 µg/ml	0.13	5 µg/ml	0.14	10 µg/ml	0.23
2	20 µg/ml	0.27	10 µg/ml	0.28	20 µg/ml	0.45
3	30 µg/ml	0.42	15µg/ml	0.41	30 µg/ml	0.69
4	40 µg/ml	0.56	20µg/ml	0.55	40 µg/ml	0.89
5	50 µg/ml	0.69	25 µg/ml	0.70	50 µg/ml	1.05

Table No 3.Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate

Replicate	Abs of Raltegravir at 328 nm	Conc of Raltegravir µg/ml	Abs of Emtricitabine at 241nm	Conc of Emtricitabine µg/ml	Abs of Tenofovir Disoproxil Fumarate at 259 nm	Conc of tenofovir disoproxil fumarate µg/ml
1	0.270	19.96 µg/ml	0.550	19.98 µg/ml	0.450	19.99
2	0.275	20.33 µg/ml	0.551	20.01 µg/ml	0.448	19.90
3	0.271	20.03 µg/ml	0.554	20.12 µg/ml	0.451	20.03
4	0.272	20.11 µg/ml	0.552	20.05 µg/ml	0.455	20.21
5	0.271	20.03 µg/ml	0.551	20.01 µg/ml	0.453	20.12
	Mean	**20.092**	***Mean***	**20.034**	***Mean***	**20.05**
	Standard Deviation	**0.143248**	***Standard Deviation***	**0.054129**	***Standard Deviation***	**0.119373**
	%RSD	**0.71%**	***%RSD***	**0.21%**	***%RSD***	**0.59%**

Table No 4 Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate

Drugs	Raltegravir			Emtricitabine			Tenofovir Disoproxil Fumarate		
Replicate	**Abs at 328 nm**	**Conc Obtained µg/ml**	**% Assay**	**Abs at 241 nm**	**Conc Obtained µg/ml**	**% Assay**	**Abs at 259nm**	**Conc Obtained µg/ml**	**% Assay**
1	0.270	19.96 µg/ml	99.8	0.550	19.98 µg/ml	99.9	0.450	19.99 µg/ml	99.95
2	0.275	20.33 µg/ml	101.65	0.551	20.01 µg/ml	100.05	0.448	19.90 µg/ml	99.50
3	0.271	20.03 µg/ml	100.15	0.554	20.12 µg/ml	100.6	0.451	20.03 µg/ml	100.15
4	0.272	20.11 µg/ml	100.55	0.552	20.05 µg/ml	100.25	0.455	20.21 µg/ml	101.05
5	0.271	20.03 µg/ml	100.15	0.551	20.01 µg/ml	100.05	0.453	20.12 µg/ml	100.6
6	0.270	19.96 µg/ml	99.8	0.550	19.98 µg/ml	99.9	0.450	19.99 µg/ml	99.95

CONCLUSION

This simultaneous equation method requires measurement of absorbance of all the three drugs at 328nm, 241 nm and 259 nm and few simple manual calculations by using simultaneous equations. The above proposed UV methods are very simple, precise, accurate, rapid and cost effective for the simultaneous estimation of Raltegravir, Emtricitabine and Tenofovir Disoproxil Fumarate from its pharmaceutical pure drugs. Hence it can be utilized for routine analysis in pharmaceutical pure drugs

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