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Research Article

RP - HPLC Method Development and Validation for Simultaneous Estimation of Emtriacitabine, Efavirenz and Tenofovir Disproxil Fumarate in Pharmaceutical Dosage Forms

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ABSTRACT

A simple new spectrophotometric method has been developed for estimation of Tenofovir disoproxil fumarate in bulk and tablet dosage form. Tenofovir disoproxil fumarate is estimated to be 261 nm in triple distilled water. The Beer's law is obeyed in the concentration range of 5 - 90 μg/ml of the drug. The slope and intercept values are 0.0109 and 0.1075, respectively. Results of analysis of this method have been validated statically and by recovery studies. The method is applied to the marketed tablet formulation. A result of the analysis of tablet formulation, given as a percentage of label claim ± standard deviation is 98.15 ± 0.76. The precision and accuracy has been examined by performing recovery studies and found to be 100.06 ± 1.24. The developed method is simple, sensitive, and reproducible, and can be used for the routine analysis of Tenofovir disoproxil fumarate in bulk and tablet dosage form.

Key words: HPLC, Tenofovir disoproxil fumarate, Emtriacitabine and Efavirenz.

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CHEMISTRY RESEARCH ARTICLE

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ABSTRACT

A simple new spectrophotometric method has been developed for estimation of Tenofovir disoproxil fumarate in bulk and tablet dosage form. Tenofovir disoproxil fumarate is estimated to be 261 nm in triple distilled water. The Beer's law is obeyed in the concentration range of 5 - 90 µg/ml of the drug. The slope and intercept values are 0.0109 and 0.1075, respectively. Results of analysis of this method have been validated statically and by recovery studies. The method is applied to the marketed tablet formulation. A result of the analysis of tablet formulation, given as a percentage of label claim ± standard deviation is 98.15 ± 0.76. The precision and accuracy has been examined by performing recovery studies and found to be 100.06 ± 1.24. The developed method is simple, sensitive, and reproducible, and can be used for the routine analysis of Tenofovir disoproxil fumarate in bulk and tablet dosage form.

Key words: HPLC, Tenofovir disoproxil fumarate , Emtriacitabine and Efavirenz.

1. INTRODUCTION

Analytical chemistry is a mean of measuring the chemical composition of natural and artificial materials. The techniques of this science are used to identify the substances which may be present in a material and to determine the exact amounts of the identified substances.

In medicine, analytical chemistry is the basis for clinical laboratory tests which help physicians diagnose disease and chart progress in recovery. In industry, analytical chemistry provides the means of testing raw materials and for assuring the quality of finished products whose chemical composition is critical. Environmental quality is often evaluated by testing for suspected contaminants using the techniques of analytical chemistry. Modern analytical chemistry is dominated by instrumental analysis. There are so many different types of instruments today that it can seem like a confusing array of acronyms rather than a unified field of study.

Analytical chemistry research is largely driven by performance (sensitivity, selectivity, robustness, linear range, accuracy, precision, and speed), and cost (purchase, operation, training, time, and space).

Pharmaceutical analysis is a specialized branch of analytical chemistry; it plays a vital role in the Quality Assurance and Quality control of pharmaceuticals. Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc.

2. SCOPE OF PRESENT WORK

The antiretroviral drugs do not cure the HIV infection; they only temporarily suppress viral replication and improve symptoms. They have various adverse effects and patients receiving these drugs require careful monitoring by adequately trained health professionals. For these reasons, continued rigorous promotion of measures to prevent new infections is essential and the need for this has not been diminished in any way by the addition of antiretroviral drugs. Adequate resources and trained health professionals are a prerequisite for the introduction of this class of drugs. Effective therapy requires commencement of three or four drugs simultaneously, and alternative regimens are necessary to meet specific requirements at start-up, to substitute for first-line regimens in the case of toxicity, or to replace failing regimens. The use of fixed-dose combinations can help simplify treatment, facilitate storage and distribution, and improve patients' adherence to the treatment plan.

The introduction of potent antiretroviral agents and the combined use of these drugs have markedly reduced the replication of HIV in many patients, and improved survival rates. The primary goals of antiretroviral therapy are to reduce HIV-related morbidity and mortality, improve quality of life, restore and preserve immunologic function, and maximally and durably suppress viral load. Plasma viremia is a strong prognostic indicator of HIV disease progression. Reductions in plasma viremia achieved with antiretroviral therapy account for substantial clinical benefits. Therefore, suppression of plasma viremia as much as possible for as long as possible is a critical goal of antiretroviral therapy.

The scope of developing and validating an analytical method is to ensure a suitable method for a particular analyze more specific, accurate and precise. The main objective for that is to improve the conditions and parameters, which should be followed in the development and validation.

Tenofovir disoproxil fumarate(Tenofovir DF):

Tenofovir is a derivative of adenosine monophosphate lacking a complete ribose ring and is the only nucleotide analog currently marketed for the treatment of HIV infection. Because the parent compound had very poor oral bioavailability, tenofovir is available only as the disoproxil fumarate prodrug, which has improved oral absorption and cellular penetration substantially. Like lamivudine and emtriacitabine, tenofovir is active against HIV-1, HIV-2, (Human immunodeficiency viruses) and HBV (Hepatitis B virus). The IC_{50} of tenofovir disoproxil fumarate against laboratory strains of HIV-1 ranges from 2 to 7 nm, making the prodrug about one hundredfold more active in vitro than the parent compound.

Description: Tenofovir DF is a white to off-white crystalline powder. The partition coefficient for tenofovir disoproxil is1.25 and the pKa is 3.75.

Solubility: Practically soluble in water and methanol.

Storage : Store in a cool place protected from light.

Mechanism of Action: Tenofovir disoproxil fumarate is hydrolyzed rapidly to tenofovir and then is phosphorylated by cellular kinases to its active metabolite, tenofovir diphosphate. The active moiety is, in fact, a triphosphate compound because the parent drug starts out as the monophosphate. The intracellular diphosphate is a competitive inhibitor of viral reverse transcriptases and is incorporated into HIV DNA to cause chain termination because it

has an incomplete ribose ring. Although tenofovir diphosphate has broad-spectrum activity against viral DNA polymerases, it has low affinity for human DNA polymerases, which is the basis for its selective toxicity.

Resistance: Virus replication in the presence of suboptimal concentrations of drug can select for mutations conferring resistance to tenofovir. Specific resistance occurs with a single substitution at codon 65 of reverse transcriptase (K65R). This mutation reduces in vitro sensitivity by only three- to fourfold but has been associated with clinical failure of tenofovir-containing regimens. Tenofovir sensitivity and virologic efficacy also are reduced in patients harboring HIV isolates with high-level resistance to zidovudine or stavudine, specifically those having three or more Thymidine analog mutations (TAMs), including M41L or L120W.

However, HIV variants that are resistant to zidovudine show only partial resistance to tenofovir, possibly a reflection of the much less efficient excision of tenofovir diphosphate by pyrophosphorolysis. The M184V mutation associated with lamivudine or emtricitabine resistance partially restores susceptibility in tenofovir-resistant HIV harboring the K65R mutation.

The K65R mutation was reported in only 2% to 3% of tenofovir-treated patients in initial clinical studies, and this mutation usually was not associated with treatment failure. Patients failing most tenofovir-containing regimens are more likely to harbor genotypic resistance to the other drugs in the regimen. Notable exceptions are once-daily combination regimens of three nucleosides, specifically tenofovir plus didanosine and lamivudine and tenofovir plus abacavir and lamivudine. Both these regimens were associated with very high early rates of virologic failure or nonresponse, and at the time of failure, the K65R mutation was present in 36% to 64% of virus isolated from patients participating in these trials.

Absorption, Distribution, and Elimination: Tenofovir disoproxil fumarate has an oral bioavailability of 25%. A high-fat meal increases the oral bioavailability to 39%, but the drug can be taken without regard to food. Tenofovir is not bound significantly to plasma proteins. The plasma elimination half-life ranges from 14 to 17 hours. The reported half-life of intracellular tenofovir diphosphate is 11 hours in activated peripheral blood mononuclear cells and 49 hours or longer in resting cells. The drug therefore can be dosed once daily. Tenofovir undergoes both glomerular filtration and active tubular secretion. Between 70% and 80% of an intravenous dose of tenofovir is recovered unchanged in the urine. Doses should be decreased in those with renal insufficiency.

Untoward Effects: Tenofovir generally is well tolerated, with few significant adverse effects reported except for flatulence. In placebo-controlled, double-blinded trials, the drug had no other adverse effects reported more frequently than with placebo after treatment for up to 24 weeks; tenofovir was significantly less toxic than stavudine. However, rare episodes of acute renal failure and Fanconi syndrome have been reported with tenofovir, and this drug should be used with caution in patients with preexisting renal disease. Because tenofovir also has activity against HBV and may lower plasma HBV DNA concentrations, caution is warranted in using this drug in patients co infected with HBV; discontinuation of tenofovir may be associated with a rebound of HBV replication and exacerbation of hepatitis.

Drug Interactions and Precautions: Tenofovir is not metabolized to a significant extent by CYPs and is not known to inhibit or induce these enzymes. However, tenofovir has been associated with a few potentially important pharmacokinetic drug interactions. A 300-mg dose of tenofovir increased the didanosine AUC by 44% to 60% probably as a consequence of inhibition of the enzyme purine nucleoside phosphorylase by both tenofovir and tenofovir monophosphate. These two drugs probably

should not be used together, or if this is essential, the dose of didanosine should be reduced from 400 to 250 mg/day. In addition, low-dose ritonavir (100 mg twice daily) increases the tenofovir AUC by 34%, and atazanavir increases the tenofovir AUC by 25%. The mechanism of these interactions is unknown.

Therapeutic Use: Tenofovir is FDA approved for treating HIV infection in adults in combination with other antiretroviral agents. The use of tenofovir in antiretroviral-experienced patients resulted in a further sustained decrease in HIV plasma RNA concentrations of 4.5 to 7.4 times relative to placebo after 48 weeks of treatment. Several large trials have confirmed the antiretroviral activity of tenofovir in three-drug regimens with other agents, including other nucleoside analogs, protease inhibitors, and/or NNRTIs.

In a randomized, double-blind comparison trial in which treatment-naive patients also received lamivudine and efavirenz, tenofovir 300 mg once daily was as effective and less toxic than stavudine 40 mg twice daily.

Efavirenz: Efavirenz is a non nucleoside reverse transcriptase inhibitor (NNRTI) with potent activity against HIV-1. The in vitro IC_{50} of this drug ranges from 3 to 9 nM. Like other compounds in this class, efavirenz does not have significant activity against HIV-2 or other retroviruses**.**

Description: Efavirenz is a white to slightly pink crystalline powder

Solubility: Practically soluble in methanol and insoluble in water.

Storage: Store in a cool place protected from light.

Mechanism of Action: Efavirenz is a noncompetitive inhibitor that binds to a site on the HIV-1 reverse transcriptase that is distant from the active site, thus inducing a conformational change that disrupts catalytic activity.

Resistance: Because the target site is HIV-1-specific and is not essential for the enzyme, resistance can develop rapidly. The most common resistance mutation seen clinically is at codon 103 of reverse transcriptase (K103N), and this decreases susceptibility up to one hundredfold or greater. Additional resistance mutations have been seen at codons 100, 106, 108, 181, 188, 190, and 225, but either the K103N or Y181C mutation is sufficient to produce clinical treatment failure. Cross-resistance extends to all FDA-approved NNRTIs.

Absorption, Distribution, and Elimination: Efavirenz is well absorbed from the gastrointestinal tract and reaches peak plasma concentrations within 5 hours. There is diminished absorption of the drug with increasing doses. Bioavailability (AUC) is increased by 22% with a high-fat meal.

Efavirenz is more than 99% bound to plasma proteins and, as a consequence, has a low CSF-plasma ratio of 0.01. The clinical significance of this low CNS penetration is unclear, especially since the major toxicities of efavirenz involve the CNS. It is recommended that the drug be taken initially on an empty stomach at bedtime to reduce side effects. Efavirenz is cleared via oxidative metabolism, mainly by CYP2B6 and to a lesser extent by CYP3A4. The parent drug is not excreted renally to a significant degree. Efavirenz is cleared slowly, with an elimination half-life of 40 to 55 hours at steady state. This safely allows once-daily dosing.

Untoward Effects: Rash occurs frequently with efavirenz, in up to 27% of adult patients. Rash usually occurs within the first few weeks of treatment and rarely requires drug discontinuation. Life-threatening skin eruptions such as Stevens-Johnson syndrome have been reported during postmarketing experience with efavirenz but are rare.

The most important adverse effects of efavirenz involve the CNS. Up to 53% of patients report some CNS or psychiatric side effects, but fewer than 5% discontinue the drug for this reason. CNS symptoms may occur with the first dose and may last for hours. More severe symptoms may require weeks to resolve. Other side effects reported with efavirenz include headache, increased hepatic transaminases, and elevated serum cholesterol. False-positive urine screening tests for marijuana metabolites also can occur depending on the assay used.

Efavirenz is the only antiretroviral drug that is unequivocally teratogenic in primates. When efavirenz was administered to pregnant cynomolgus monkeys, 25% of fetuses developed malformations. In six cases where women were exposed to efavirenz during the first trimester of pregnancy, fetuses or infants had significant malformations, mainly of the brain and spinal cord. Women of childbearing potential therefore should use two methods of birth control and avoid pregnancy while taking efavirenz.

Drug Interactions and Precautions: Efavirenz is a moderate inducer of hepatic enzymes, especially CYP3A4. It undergoes limited autoinduction, but because of its long half-life, there is no need to alter drug dose during the first few weeks of treatment. Efavirenz decreases concentrations of phenobarbital, phenytoin, and carbamazepine; the methadone AUC is reduced by 33% to 66% at steady state. Rifampin concentrations are unchanged by concurrent efavirenz, but rifampin may reduce efavirenz concentrations. Efavirenz reduces the rifabutin AUC by 38% on average. Efavirenz has a variable effect on HIV protease inhibitors. Indinavir, saquinavir, and amprenavir concentrations are reduced, but ritonavir and nelfinavir concentrations are increased. Drugs that induce CYP2B6 or CYP3A4 (e.g., phenobarbital, phenytoin, and carbamazepine) would be expected to increase the clearance of efavirenz and should be avoided.

Therapeutic Use: Efavirenz was the first antiretroviral agent approved by the FDA for once-daily administration. Initial short-term monotherapy studies showed substantial decreases in plasma HIV RNA, but the drug should only be used in combination with other effective agents and should not be added as the sole new agent to a failing regimen. In antiretroviral-naive patients receiving efavirenz, zidovudine, and lamivudine, 70% achieved undetectable plasma HIV-1 RNA compared with 48% of those receiving indinavir plus zidovudine and lamivudine. Much of this difference appeared to be the consequence of improved patient adherence to the efavirenz regimen. Efavirenz also has been used effectively in patients who have failed previous antiretroviral therapy in combination with other active drugs. In pediatric HIV infection, 60% of children failing prior therapy with a nucleoside reverse transcriptase inhibitor had sustained virologic benefit after 48 weeks of treatment with efavirenz, nelfinavir, and a nucleoside analog.

Emtricitabine: Emtricitabine is a cytosine analog that is chemically related to lamivudine and shares many of that drug's pharmacodynamic properties. Like lamivudine, it has two chiral centers and is manufactured as the enantiomerically pure (2R, 5S)-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (FTC). Emtricitabine is active against HIV-1, HIV-2, and HBV. The IC_{50} of emtricitabine against laboratory strains of HIV-1 ranges from 2 to 530 nM, although, on average, the drug is about 10 times more active in vitro than lamivudine.

Description: Emtricitabine is a white to off-white crystalline powder

Solubility: Practically soluble in water and methanol.

Storage: Store in a cool place protected from light.

Mechanism of Action: Emtricitabine enters cells by passive diffusion and is phosphorylated by deoxycytidine kinase and cellular kinases to its active metabolite, emtricitabine triphosphate. The intracellular triphosphate acts as a competitive inhibitor of reverse transcriptase and is incorporated into HIV DNA to cause chain termination. Like lamivudine, emtricitabine has low affinity for human DNA polymerases, explaining its low toxicity to the host.

Resistance: High-level resistance to emtricitabine occurs with the same mutation (methionine-to-valine substitution at codon 184) affecting lamivudine, although this appears to occur less frequently with emtricitabine. In three studies, M184V occurred about half as frequently with emtricitabine-containing regimens as with lamivudine, and patients presenting with virologic failure were two to three times as likely to have wild-type virus at the time of failure as compared with lamivudine. The M184V mutation restores zidovudine susceptibility to zidovudine-resistant HIV and also partially restores tenofovir susceptibility to tenofovir-resistant HIV harboring the K65R mutation.

Absorption, Distribution, and Elimination:

Emtricitabine is absorbed rapidly and has an oral bioavailability of 93%. Food reduces the C_{max} but does not affect the AUC, and the drug can be taken without regard to meals. Emtricitabine is not bound significantly to plasma proteins. Compared with other nucleoside analogs, the drug has a slow systemic clearance and long elimination half-life of 8 to 10 hours. In addition, the estimated half-life of the intracellular triphosphate is very long, up to 39 hours in one report. This provides the pharmacokinetic rationale for once-daily dosing of this drug. Emtricitabine is excreted primarily unchanged in the urine, undergoing glomerular filtration and active tubularsecretion.

Untoward Effects: Emtricitabine is one of the least toxic antiretroviral drugs and, like its chemical relative lamivudine, has few significant adverse effects and no effect on mitochondrial DNA in vitro. Prolonged exposure has been associated with hyperpigmentation of the skin, especially in sun-exposed areas. Elevated hepatic transaminases, hepatitis, and pancreatitis have been reported, but these have occurred in association with other drugs known to cause these toxicities. Since emtricitabine also has in vitro activity against HBV, caution is warranted in using this drug in patients coinfected with HBV; discontinuation of lamivudine, which is closely related to emtricitabine, has been associated with a rebound of HBV replication and exacerbation of hepatitis.

Drug Interactions and Precautions: Emtricitabine is not metabolized to a significant extent by CYPs, and it is not susceptible to any known metabolic drug interactions. The possibility of a pharmacokinetic interaction involving renal tubular secretion, such as that between trimethoprim and lamivudine, has not been investigated for emtricitabine, although the drug does not alter the pharmacokinetics of tenofovir.

Therapeutic Use: Emtricitabine is FDA approved for treating HIV infection in adults in combination with other antiretroviral agents. Two small monotherapy trials showed that the maximal antiviral effect of emtricitabine (mean 1.9 log unit decrease in plasma HIV RNA concentration) was achieved with a dose of 200 mg/day. Several large trials have confirmed the antiretroviral activity of emtricitabine in three-drug regimens with other agents, including nucleoside or nucleotide analogs, protease inhibitors, and/or NNRTIs. In two randomized comparison studies, emtricitabine- and lamivudine-based triple-combination regimens had similar efficacy.

The selection of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and

partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase.

Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reverse phase columns are recommended. The Preferred experimental conditions for the initial HPLC separation are given in Table 2.4.

Using these conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable.

If typical reverse-phase conditions provided inadequate sample retention, it suggests the use of either ion-pair or normal phase HPLC. Alternatively, the sample may be strongly retained with 100% ACN as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

3. METHOD OF DEVELOPMENT

The objective of this experiment was to optimize the assay method for simultaneous estimation of Tenofovir, Efavirenz and Emtricitabine based on the literature survey made and the methods given in official pharmacopoeias. So here the trials mentioned describes how the optimization was done.

Trial: 1

Buffer preparation: weigh accurately 2.8 gms of di sodium hydrogen ortho phosphate & 2g hexon sulphuric acid were dissolve into 1000 ml of Milli-Q water & filter through 0.45µm nylon membrane filter and degas.

Mobile phase-A: Buffer and methanol were mixed in the ratio of 95:5 and sonicated to degas.

Mobile phase-B: : Buffer and ACN were mixed in the ratio of 40:60(v/v) and sonicated to degas.

Chromatographic conditions:

Flow rate : 1.0 ml/min

Column : Hypersil BDF C18, 150 x 4.6 mm, 5µ

Detector wave length : 270nm

Column temperature : Ambient

Injection volume : 10µl

Run time : 30 mins

Diluent : Mobile phase A

Observation:

S.NO	Name of the peak	Retention time(min)
1.	Efavirenz	25.455
2.	Tenofovir	13.616
3.	Emtricitabine	2.097

Conclusion: The retention time was too long for Efavirenz and the peaks were non symmetric

Trial: 2

The buffer, mobile phase-A and mobile phase-B preparations were similar to that of Trial: 1.

Chromatographic conditions:

Flow rate : 1.2 ml/min

Column : Altima C18, 150 x 4.6 mm, 5µ

Detector wave length : 260nm

Column temperature : Ambient

Injection volume : 20µl

Run time : 25 mins

Diluent : Mobile phase

S.NO	Name of the peak	Retention time(min)
1.	Efavirenz	20.935
2.	Tenofovir	12.684
3.	Emtricitabine	2.187

Conclusion: The peak of Tenofovir was doesn't passes USP tailing factor i.e 2.4.

Trial: 3

Buffer preparation: weigh accurately 4.6g of Ammonium Acetate and dissolve it in 1000ml of Milli-Q water. Adjust the pH to 4.6 with Glacial acetic acid, filter through 0.45µm nylon membrane filter and degas.

Mobile phase-A: pH 4.6 buffer

Mobile phase-B: Acetonitrile

Chromatographic conditions:

Flow rate : 2.0 ml/min

Column : Xterra RP-18, 150 x 4.6 mm, 5µ

Detector wave length : 260nm

Column temperature : Ambient

Injection volume : 20µl

Run time : 20 mins

Diluent : Methanol

Observation:

S.NO	Name of the peak	Retention time(min)
1.	Efavirenz	18.766
2.	Tenofovir	11.857
3.	Emtricitabine	2.327

Tenofovir Standard stock Preparation: Weigh and transfer accurately about 60.0 mg of Tenofovir disoproxil fumerate Working Standard into a 100 ml clean dry volumetric flask, add about 60 ml of methanol, sonicate for 5 minutes, and dilute to volume with methanol.

Efavirenz Standard stock Preparation: Weigh and transfer accurately about 60.0 mg of Efavirenz Working Standard into a 100 ml clean dry volumetric flask, add about 60 ml of methanol, sonicate for 5 minutes, and dilute to volume with methanol.

Emtricitabine Standard stock Preparation: Weigh and transfer accurately about 40.0 mg of Emtricitabine Working Standard into a 100 ml clean dry volumetric flask, add about 60 ml of methanol, sonicate for 5 minutes, and dilute to volume with methanol.

Diluted Standard: Pipette out 5 ml of the Efavirenz standard stock solution, 5ml of Emtricitabine Standard stock solution and 5ml Tenofovir Standard stock solution and dilute to 50 ml with diluent.

Sample preparation: Weigh five tablets and transfer into a 500ml volumetric flask. Add about 100ml of buffer and shake the volumetric flask on a rotary shaker for 20min, add 200ml of methanol and sonicate for 20min with intermittent shaking and dilute to volume with methanol&mix. From this pipette out 2ml of sample solution into a 100ml volumetric flask,

make up the volume with diluent and filter the solution through 0.45µ nylon membrane filter to obtain clear solution.

Evaluation of System Suitability: Inject 20 µl of the diluted standard solution in five replicate injections, into the chromatograph and record the chromatograms.

The column efficiency as determined from Tenofovir, Efavirenz and Emtricitabine peaks is not less than 5000 USP plate count and the tailing factor for Tenofovir, Efavirenz and Emtricitabine peaks is not more than 2.0. The relative standard deviation for the peak areas of the five replicate injections is not more than 2.0%.

Procedure: Flush the HPLC system thoroughly with water followed by methanol. Equilibriate the column for NLT 30min with intial mobile phase composition at a flow rate of 0.8ml/min.

Separately inject 20µl of the blank, Standard (five injections) and sample solution in duplicate into the liquid chromatography, record the chromatographs and measure the peak areas.

4. METHOD OF VALIDATION

SYSTEM SUITABILITY: A Standard solution was prepared by using Tenofovir disoproxil fumerate, Efavirenz and Emtricitabine working standards as per test method and was injected ten times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from ten replicate injections for Tenofovir, Efavirenz and Emtricitabine retention times and peak areas.

ACCEPTANCE CRITERIA:

1. The % RSD for the retention times of principal peak from 10 replicate injections of each Standard solution should be not more than 2.0 %

2. The % RSD for the peak area responses of principal peak from 10 replicate injections of each standard Solution should be not more than 2.0%.

3. The number of theoretical plates (N) for the Tenofovir, Efavirenz and Emtricitabine peaks is NLT 3000.

4. The Tailing factor (T) for the Tenofovir, Efavirenz and Emtricitabine peaks is NMT .

OBSERVATION:

The %RSD for retention times and peak areas were found to be within the limits. Refer tables 5.1a, 5.1b and 5.1c.

ACCURACY (RECOVERY):

A study of Accuracy was conducted. Drug Assay was performed in triplicate as per test method with equivalent amount of Tenofovir, Efavirenz and Emtricitabine into each volumetric flask for each spike level to get the concentration of Tenofovir, Efavirenz and Emtricitabine equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of Tenofovir, Efavirenz and Emtricitabine was calculated.

Separately inject the blank, placebo, Tenofovir, Efavirenz and Emtricitabine in to the chromatograph.

ACCEPTANCE CRITERIA:

The mean % recovery of the Tenofovir, Efavirenz and Emtricitabine at each level should be not less than 95.0% and not more than 105.0%.

I) EMTRICITABINE -- TABLE-5.1A

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	8.487	1452576	81876	1.01
2	8.474	1474749	81768	1.00
3	8.474	1456080	81975	1.00
4	8.510	1464908	83007	1.00
5	8.471	1462029	82375	1.00
6	8.460	1460847	81767	1.00
7	8.472	1459513	82788	1.00
8	8.462	1480078	82423	1.00
9	8.486	1475716	82507	1.00
10	8.476	1477837	84017	1.00
Mean	8.476	1466433	82450	1.00
SD	0.0152	9840.84	…	---
% RSD	0.18	0.67	….	---

II) TENOFOVIR : TABLE-5.1B

Injection	RT(min)	Peak Area	USP Plate count	USP Tailing
1	16.776	1761685	188018	1.16
2	16.754	1786938	188836	1.15
3	16.753	1767328	189703	1.15
4	16.737	1775282	190421	1.15
5	16.747	1770630	192623	1.14
6	16.739	1769621	193100	1.14
7	16.752	1769174	194710	1.14
8	16.735	1792952	194327	1.14
9	16.732	1786536	194522	1.14
10	16.757	1788633	194900	1.15
Mean	16.748	1776878	192116	1.15
SD	0.0132	10886.61	---	….
% RSD	0.08	0.61	---	---

III) EFAVIRENZ: TABLE-5.1C

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	20.984	649875	187674	0.99
2	20.949	659438	188480	0.99
3	20.937	652264	189268	0.99
4	20.956	656257	191566	0.99
5	20.919	656652	192402	0.98
6	20.906	657415	193577	0.98
7	20.923	656708	193749	0.98
8	20.930	663763	193845	0.98
9	20.912	660562	193354	0.98
10	20.941	659215	193008	0.98
Mean	20.936	657152	191692	0.98
SD	0.0233	3999.11	…..	---
% RSD	0.11	0.61	…..	---

ACCURACY OF TENOFOVIR --TABLE-5.4a

Concentration% of spiked level/Sample ID	Amount added(mg)	Amount found(mg)	% Recovery	Statistical Analysis of % Recovery	
50%Sample 1	750.88	758.00	100.9	MEAN	100.8
50%Sample 2	751.16	756.37	100.7	SD	0.12
50%Sample 3	749.32	755.83	100.9	%RSD	0.12
100 %Sample 1	1499.88	1507.59	100.5	MEAN	100.2
100%Sample 2	1500.38	1501.35	100.1	SD	0.23
100%Sample 3	1499.17	1500.63	100.1	%RSD	0.23
150%Sample 1	2250.76	2259.52	100.4	MEAN	100.3
150%Sample 2	2250.76	2257.41	100.3	SD	0.10
150%Sample 3	2250.48	2255.87	100.2	%RSD	0.10

ACCURACY OF EFAVIRENZ -- TABLE-5.4b

Concentration% of spiked level/sampleID	Amount added(mg)	Amount found(mg)	% Recovery	Statistical Analysis of % Recovery	
50%Sample 1	1497.41	1523.07	101.7	**MEAN**	101.4
50%Sample 2	1498.51	1515.52	101.1	**SD**	0.31
50%Sample 3	1497.84	1520.88	101.5	**%RSD**	0.31
100 %Sample 1	2987.80	3003.50	100.5	**MEAN**	100.6
100%Sample 2	2989.08	3005.84	100.6	**SD**	0.06
100%Sample 3	2987.43	3003.92	100.6	**%RSD**	0.06
150%Sample 1	4482.72	4483.04	100.0	**MEAN**	100.0
150%Sample 2	4482.99	4482.06	100.0	**SD**	0.00
150%Sample 3	4480.95	4481.82	100.0	**%RSD**	0.00

ACCURACY OF EMTRICITABINE -- TABLE-5.4c

Concentration% of spiked level/sampleID	Amount added(mg)	Amount found(mg)	% Recovery	Statistical Analysis of % Recovery	
50%Sample 1	499.42	506.51	101.4	**MEAN**	101.3

For sample (over all % RSD)

	0.9ml/mn	0.13	0.33	0.10	1.00	1.04	1.09	172815	51286	150647
Temp. variation (ambient)	30ºC	0.21	0.19	0.18	0.99	1.06	1.09	162766	56635	157173
pH of mobile phaseA-4.6	4.4	0.74	0.99	1.18	0.99	1.05	1.10	155895	55972	152167
	4.8	1.15	1.65	1.59	1.00	1.04	1.10	153501	58058	150511

EFAVERINZ (TABLE 5.8B)

parameter		Assay (% labeled amount)			Over all %RSD
Flow variation	0.7ml/min	100.9	101.0	101.0	0.29
0.8ml/min	0.8ml/min	102.3	102.3	102.4	0.46
Temperature variation(ambient)	30ºC	101.7	101.9	102.0	0.27
pH of mobile phase A (4.6)	4.4	99	99.5	99.4	1.08
	4.8	98.7	99.3	99.1	1.22

EMTRIACITABINE (TABLE5.8C)

parameter		Assay (% labeled amount)			Over all %RSD
Flow variation	0.7ml/min	99.2	100.4	100.9	0.82
0.8ml/min	0.8ml/min	101.0	100.5	100.9	1.03
Temperature variation(ambient)	30ºC	100.6	100.3	100.5	0.84
pH of mobile phase A (4.6)	4.4	96.6	96.2	97.2	1.15
	4.8	96.3	96.4	96.9	1.20

TENOFOVIR DISPROXIL FUMARATE (TABLE5.8D)

parameter		Assay (%labelled amount)			Over all %RSD
Flow variation 0.8ml/min	0.7ml/min	100.9	101.0	101.0	0.29
	0.8ml/min	102.3	102.3	102.4	0.46
Temperature variation(ambient)	30ºC	101.7	101.9	102.0	0.27
pH of mobile phase A (4.6)	4.4	99	99.5	99.4	1.08
	4.8	98.7	99.3	99.1	1.22

5. VALIDATION REPORT

The test method is validated for Specificity, Linearity, Precision, Accuracy, Range, Stability of solution, Ruggedness and Robustness and found to be meeting the predetermined acceptance criteria. The validated method is Specific, Linear, Precise, Accurate, Robust and Rugged for the determination of assay for Efaverinz ,Emtriacitabine & Tenofovir disproxil fumarate tablets 300/600/200mg.

6. SUMMARY AND CONCLUSION

Development of new analytical methods for the determination of drugs in pharmaceutical dosage forms is more important in pharmacokinetic, toxicological and biological studies. Today pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. The pharmaceutical industry is under increased scrutiny from the government and the public interested groups to contain costs and at consistently deliver to market safe, efficacious product that fulfill unmet medical needs. The pharmaceutical analyst plays a major rule in assuring identity, safety, efficacy, purity, and quality of a drug product. The need for pharmaceutical analysis is driven largely by regulatory requirements. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications, and method validation. Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products. New methods are now being development with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

Pharmaceutical analysis occupies a pivotal role in statuary certification of drugs and their formulations either by the industry or by the regulatory authorities. In industry, the quality assurance and quality control departments play major role in bringing out a safe and effective drug or dosage form. The current good manufacturing practices (CGMP) and the Food Drug Administration (FDA) guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Therefore, the complexity of problems encountered in pharmaceutical analysis with the importance of achieving the selectivity, speed, low cost, simplicity, sensitivity, specificity, precision and accuracy in estimation of drugs.

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