Analytical method development, validation, Synthesis, Characterization and Forced degradation study of Tenofovir Disoproxil Fumarate and its impurities.

Rinchi Bora [*], M R Jeyaprakash[**], Samrat Debnath [***], S. Jubie[****]

Department of Pharmaceutical Analysis[1], Department of Pharmaceutical Chemistry[2], JSS College of Pharmacy, JSS Academy of Higher Education & Research, Ooty, Nilgiris, Tamil Nadu, India

Abstract- Tenofovir disoproxil fumarate (TDF) is a prodrug of antiretroviral class of drugs belonging to NRTIs. There are several simultaneous estimations reported for the drug tenofovir disoproxil fumarate (TDF) and different synthetic routes for synthesizing its impurity is also available. Due to cost effective method we chose to synthesize the impurity. To develop an analytical method for tenofovir disoproxil fumarate and one of its impurity. Synthesis and characterization of the impurity has to be performed. RP-HPLC with Shimpack C_{18} column (4.6 x 50 mm, i.d. 3µm) is used. A combination of methanol- acetonitrile (50:50) and ammonium acetate (pH 4.19) in the ratio of 50:50 V/V is used as the mobile phase. The flowrate is set at 1 mL/min. The absorbance was finalized using a UV-Vis spectrophotometer. LC-MS was used to obtain the mass spectra. IR was used for determining the functional group and NMR was used for the proton number. The absorbance was set at 260nm. From the analytical method development, LC showed drug peak at 6.103 and 8.621mins for the synthesized impurity. For the LC-MS, the impurity peak is seen at 1051.55. The developed method is found to be 98.85% accurate and precision obtained is 0.55%. It was found to be linear in the range of 10-60 µg/ml and passed Beer's law with a correlation coefficient of 0.999. LOD and LOQ is found to be 0.514 and 1.713 µg/ml respectively. We can conclude from the performed experiment that a novel, simple and precise analytical method has been developed and validated for tenofovir disoproxil fumarate and for one of its impurity. Synthesis of the impurity has also been performed and then characterization of the same.

Index Terms- Tenofovir Disoproxil Fumarate, RP-HPLC, LC-MS, Acetonitrile, Impurities.

INTRODUCTION

Tenofovir disoproxil fumarate ([[(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-(propan-2-yloxycarbonyloxymethoxy)phosphoryl]oxymethyl propan-2-yl carbonate;but-2-enedioic acid) is the prodrug of tenofovir, an antiretroviral drug belonging to the NRTIs sub-discip. It prevents viral DNA from replicating, which is how it functions. In antiretroviral therapy (ART) missions, this drug is used in combination with other drugs. $C_{23}H_{34}N_5O_{14}P$ is the chemical formula. It is 635.5 g/mol in molecular weight. It was confirmed to have some impurities, but it was omitted from the most recent version of the pharmacopoeias. There are no indications that they were removed due to lack of evidence.[1]

In 2001, the United States Food and Drug Administration (USFDA) approved it for the treatment of HIV. It was later expanded to include pediatrics in the year 2012. In 2015, it was also approved for the treatment of chronic HBV (Hepatitis B virus). Tenofovir disoproxil fumarate (tenofovir DF) has been linked to severe and potentially fatal side effects. Lactic acidosis, serious liver complications, and new or worsening kidney problems, including kidney failure, are examples of these.[2]

Obtaining a reference standard for impurities to monitor and preserve the consistency of active pharmaceutical ingredients is extremely difficult (API). There are numerous papers available in this regard, in which the synthesis of the same has been carried out for cost-cutting purposes and in which no in-depth analysis of the impurities has been reported.

According to the latest updated winning scenario, about 21 lakh people are infected with HIV, with 49 percent of them unable to access effective antiretroviral treatment (ART)[3-5]. Furthermore, an additional 1.8 million people worldwide may have recently been infected with HIV, including 1,50,000 (15 years) in the United States [6,7]. Currently, 20.9 million people living with HIV are receiving antiretroviral therapy (ART) around the world [8]. There are approximately six distinct classifications of ART instruments proposed in the current pharma field to control viral infections [9].

The drugs chosen are from the National Essential Medicine List (NLEM) and are among the most widely recommended by the WHO ARV project to eradicate HIV/AIDS from society [10-12]. The list of debasements for these drugs is compiled from Pharmacopeias available all over the world. The amount of contaminations mentioned in the various Pharmacopeias varies by country [13]. The final debasements will be selected based on the score and presence of underlying alarms that have been shown to be mutagenic/carcinogenic [14].

There are many steps to completing a study. The inquiry and the P benefit of docking score will be moved forward to an immediate and circuitous severe immunoassay to show the likelihood. The primary stage, retroviral drug QSAR study, docking score, will be compared with and without the presence of the authority monograph pollutant structure, the P benefit of docking score, and the investigation will be advanced to an immediate and circuitous severe immunoassay to demonstrate the possibility. An in vitro cell line analysis will also be performed. In addition, the in vitro cell line analysis will be used as a tool to evaluate the viability of ART in an infection cell line with and without the involvement of associated substances/contamination impacts during the reconsideration of obstruction instrument in clinical implications. Individual-level care decisions will benefit from genotypic obstruction research, yet effective models for delivering opposition testing in low- and middle-income countries have yet to be established.

The study will compare and contrast the ART mission to succeed with a valid and reduced antiretroviral drug measurement to avoid ART obstruction during clinical care.

1. Materials and Methods

1.1 Chemicals and reagents:

Mylan Pvt. Ltd., Hyderabad, sent a gift sample of tenofovir disoproxil fumarate. Carbonio.com is where you can get paraformaldehyde of analytical grade. Water for HPLC was obtained from the Milli-Q plus water purification system. Sigma–Aldrich Corporation provided HPLC grade methanol, acetonitrile, ammonium acetate, glacial acetic acid, dimethyl formamide, and dichloromethane (St. Louis, MO, USA).

1.2 Synthesis of TDF impurity:

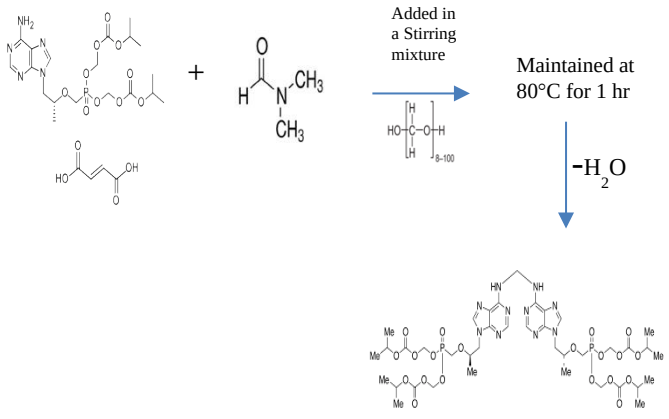


Fig 1: Schematic presentation of TDF impurity synthesis

1.3 Equipment and chromatographic conditions:

1.3.1 UV-Spectroscopy:

The wavelength of the drug and the synthesized impurities were determined using UV-Spectroscopy (UV-1700Pharmaspec, Shimadzu). Purified milli Q water was used for the experiment and all dilutions. The absorbance of the drug and the impurities were 260nm and 268nm, respectively. And the final wavelength was determined by overlapping each other by nm for the HPLC experiment. (Fig 2)

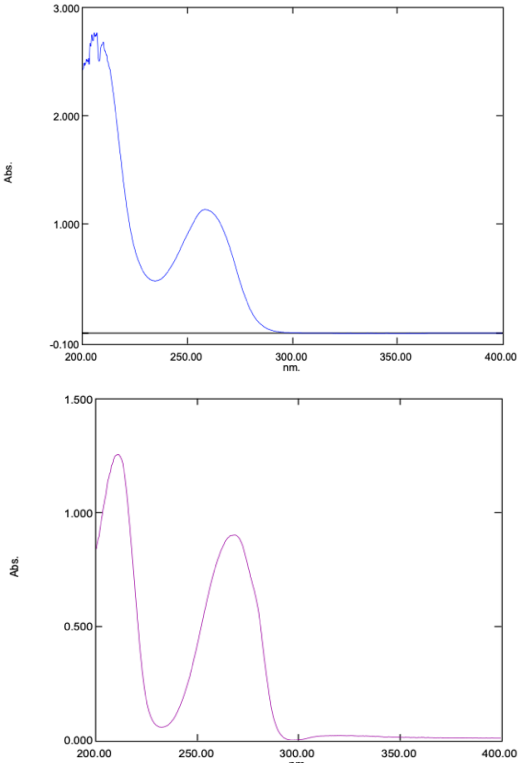
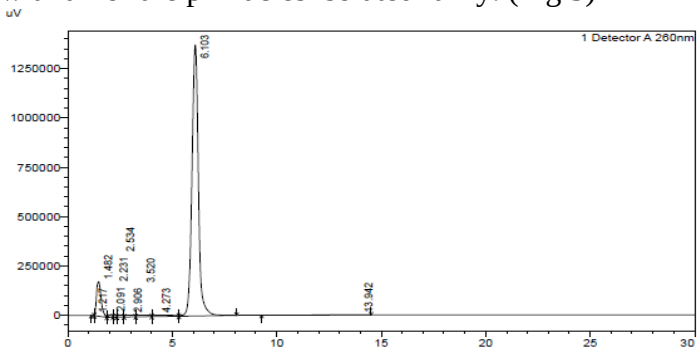


Fig 2: UV-Vis spectrum of TDF and its synthesized impurity

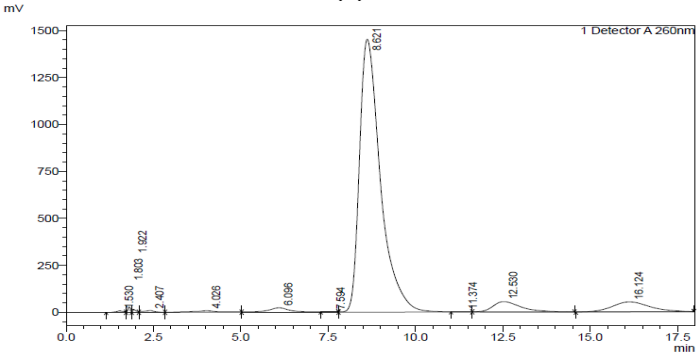
1.3.2 High performance liquid chromatography (HPLC):

The tests were performed on a Shimadzu conspicuousness SPD 20A module with a 2487 UV indicator and a shimpack C_{18} column (4.6 x 50 mm, i.d. 3µm). By varying the proportions of acetonitrile-methanol (50:50) and ammonium acetic acid derivation (pH of 4.19 with glacial acetic acid), the portable stage was set up at the proportion of 50: 50 V/V. Where the pinnacle and region were in good working order and obtained a peak at 6.103 and 8.621mins of TDF and its synthesized impurity. Lab station programming was used to record the data. The segment's temperature was maintained at room temperature. The arrangement's

flow rate is 1ml/min. The medication's maintenance season was minimal, and the pollutions were minimal, with all of the pinnacles isolated fairly. (Fig 3)



(a)



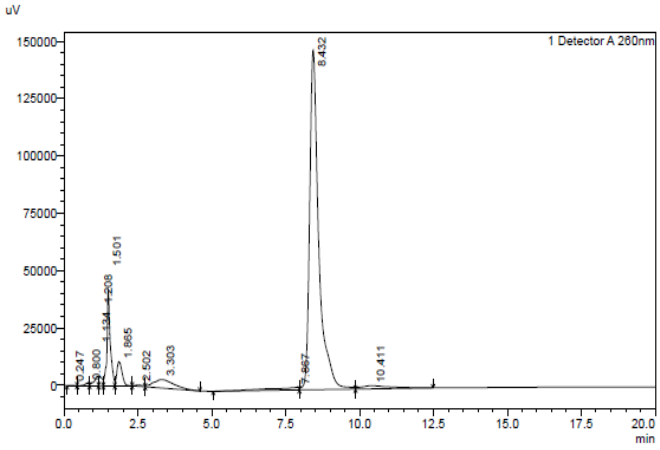
(b)

Fig 3: a. TDF chromatogram b. Synthesized TDF impurity

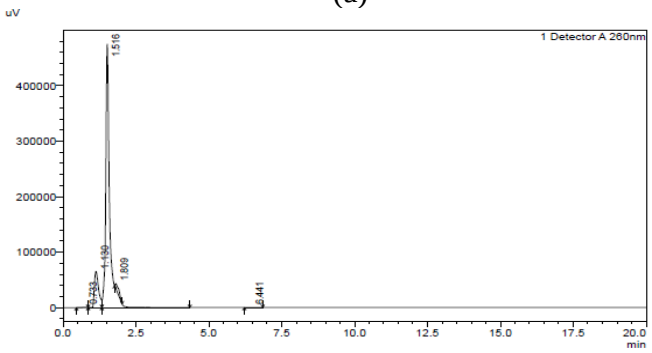
1.3.3 Degradation studies:[15-17]

According to the ICH guidelines, forced degradation studies were performed. It should be noted that more difficult conditions (25°C/60 percent RH or 40°C/75 percent RH) are used than those used for accelerated exams. At a minimum, the following conditions should be investigated: (1) corrosive and base hydrolysis, (2) hydrolysis at various pH levels, (3) warm debasement, (4) photolysis, and (5) oxidation.

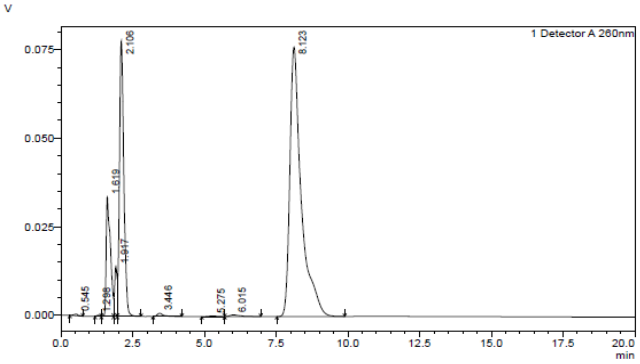
Acid, alkali, peroxide, UV, and heat were all used to degrade the medication. Stress degradation by hydrolysis under alkaline conditions using 0.1N NAOH was completely degraded in TDF force degradation experiments. Stress degradation was determined to be 10.95 % after hydrolysis in an acidic environment with 0.1N HCl. Hydrogen peroxide was used to perform oxidative degradation, which resulted in a 12.22 % reduction in product deterioration. The rate of hydrolytic breakdown in the neutral state was determined to be 12.26%. Under the given experimental settings, forced degradation experiments of the medication show high stability.



(a)



(b)



(c)

Fig 4: Degradation study chromatogram of TDF; a. acid hydrolysis, b. Base hydrolysis, c. Peroxide hydrolysis

1.3.4 Mass Spectrometry:

For research, a Shimadzu 8030 LC system with tandem quadrupole mass spectrometry.

Stationary phase	Shimpack C_{18} (4.6 x 50 mm, i.d., 3µm)
Mobile Phase	**Solvent A:** Methanol+Acetonitrile **Solvent B:** (10mM) Amm. Acetate (pH 4.19)
Ratio	30:70 % v/v

Flow rate	0.5 mL/min
Injection volume	20 µl using Manual injector
Temperature	Ambient Temperature
Detector	PD-M20 PDA
Interface	ESI
Operation mode	MRM
Polarity	Positive
Probe temperature	Ambient
DL Temperature	250 °C
Block Temperature	350 °C
CID Gas	230 Kpa
Detector voltage	1.3 kV
Nebulizer Gas flow	3 L/min
Drying gas	15 L/min
Data station	Lab solution

1.3.5 Selection of mass range:

The mass spectrometer was infused with 1000 µg/mL of TDF and its impurities, and the operating conditions were calibrated. To track impurities, transitions 1100-100 m/z were used in which 1051.55 m/z[M+H] is of the synthesized impurity (Fig.5).

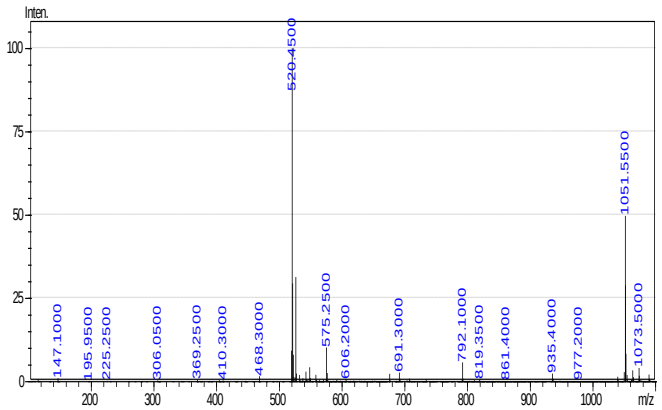


Fig 5: Mass spectrum of TDF impurity

1.3.6 Fourier transform infrared spectroscopy:

FTIR-8400S is combined with the IR solution which is having a 32-bit high performance FTIR software to analyze the samples easily and securely.

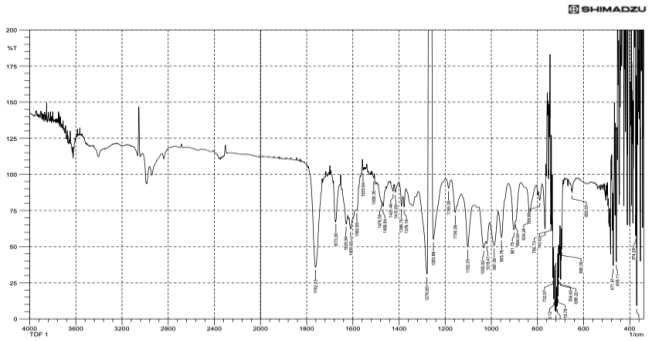


Fig 6: IR spectrum of the synthesized TDF impurity.

1.3.7 Nuclear Magnetic Resonance:

H^1 NMR (400MHz, $CDCl_3$) d 8.42 (s, 2H), 7.87 (d, J1⁄4123.7Hz, 4H), 5.76-5.48 (m, 10H), 4.91 (dq, J1⁄412.3, 6.1Hz, 4H), 4.35 (d, J1⁄413.6Hz, 2H), 4.17 (dd, J1⁄414.3, 6.9Hz, 2H), 3.95 (s, 4H), 3.75 (dd, J1⁄413.7, 9.0Hz, 2H), 1.25 (dd, J1⁄433.7, 5.3Hz, 30H).

Protonis numbered	Chemical shift (ppm)	The multiplicity * at peak	Proton number				
				1	8.42	s	2H
2	7.92	s	2H				
				3	7.87	br	2H
4	5.63-5.55	m	10H				
				5	5.76-5.48	m	4H
6	4.31-4.28	m	2H				
				7	4.91-4.17	Dd, J=1⁄414.3 and 6.9Hz	2H
8	3.90-3.89	m	4H				
				9	3.95-3.75	Dd, J1⁄413.7 and 9.0Hz	2H
10	1.27-1.24	m	30H				
				11	1.25	D, J=1⁄433.7 and 5.3Hz	6H

1.4 Method Development (18-24)

2.4.1 Preparation of working standard solution:

The TDF arrangement was made by dissolving 10 mg of TDF in 10 mL of acetonitrile-methanol and storing it at 2-8°C. Weakening the stock structure with

acetonitrile-methanol diluents yielded working arrangements.

2.4.2 Preparation of working impurities solution:

The impurities solution was made by dissolving 10 mg of impurities in 10 mL of acetonitrile-methanol and keeping it refrigerated between 2 and 8°C. Diluting the stock solution with diluent acetonitrile-methanol yielded working solutions.

2.4.3 Preparation of sample solution:

To achieve a concentration of 10 µg/mL of TDF, the weight equal to 0.10 g of TDF was measured and moved to a 100 mL volumetric flask, where it was dissolved in methanol and the amount was made up with acetonitrile.

2. Method Validation:

The method was validated for specificity, linearity, accuracy, precision, range, quantitation limit, and detection limit, robustness and system suitability as per the ICH guidelines [25-26].

2.1 Specificity:

The capacity of a tool to calculate the analyte reaction in the presence of other drugs, excipients, and their possible impurities is referred to as specificity.

2.2 Linearity:

The linearity of TDF was assessed using an 8 concentration spectrum of an average of 6 determinations, from 10 to 60 µg/mL. A calibration curve was used for the calibration of the coefficient correlation, incline and intercept value to determine the linearity of the proposed system.

2.3 Accuracy:

Recovery experiments using the traditional addition procedure, as per ICH recommendations, were used to assess the method's accuracy. The normal drug TDF was spiked into the pre-analyzed samples.

2.4 Precision:

Inter-day and intra-day precision tests were used to assess the method's precision. Samples with three concentration ranges were prepared as low (LQC), medium (MQC), and high (HQC) consistency controls at six replicates, equivalent to 10, 30, and 60 µg/mL, respectively. The accuracy was calculated using the percent relative standard deviation (percent RSD) of the regressed concentration.

2.5 Limit of detection and limit of quantification:

The signal-to-noise ratio of 3:1 was used to establish the detection limit. The signal-to-noise ratio also defined the maximum of quantification, with the drug being quantifiable with a minimum peak area of 10:1.-to-noise ratio, was the drug could be quantified with minimum peak area in the ratio of 10:1.

2.6 Robustness:

By varying the experimental conditions (operators, reagent source, and column of similar type) and optimizing the conditions, the robustness of the methods was investigated (pH, mobile phase ratio and flow rate).

2.7 **System suitability:**

System suitability test is an integral part of method development.

Table 1: System suitability

Sl. no	Parameter	Acceptance criteria	Obtained results
1	Theoretical plates	NLT 2000	8100
2	Accuracy	Recovery 98-102%	98.85%
3	Precision	% RSD NMT2	0.55%
4	Specificity	No interference	No impurity
5	Linearity		10-60 µg/ml
6	Ruggedness	% RSD NMT 2	0.691
7	LOD		0.514 µg/ml
8	LOQ		1.713 µg/ml

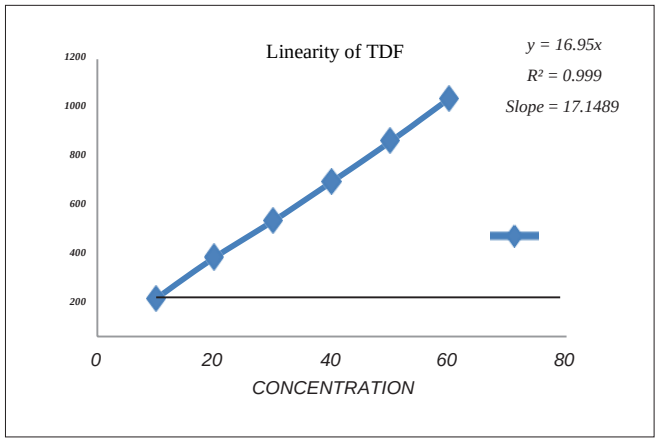
3. RESULTS AND DISCUSSION:

3.1 Specificity:

The specificity test shows that the excipients used did not conflict with the main compound's peak. Along with the TDF retention period, no peaks were eluted (Fig.3). As a result of the findings, the established method for determining TDF in formulations was found to be selective. (Table 1)

3.2 Linearity:

Six determinations at eight concentration ranges covering a spectrum of 10-60 µg/mL for TDF were used to assess the method's linearity. A calibration curve was discovered to be linear with a mean regression of equation (Y=16.95 X, r^2 0.999; Slope= 17.1489), where Y is the peak area ratio of the analyte to the impurities and X is the concentration of an analyte in µg/mL, respectively (Fig.7).



3.3 Accuracy:

he system's accuracy was tested using the traditional addition method on three quality control (LQC, MQC, and HQC) samples, and it was found to be 99.12% accurate. In the API, the built method was used to calculate TDF (Table I).

3.4 Precision:

Intra-day and inter-day precision experiments at three different concentrations were used to measure the method's precision, which were considered to be within the limits (Table I).

3.5 Limit of detection and Limit of quantification:

Based on a signal-to-noise ratio of 3:1, the method's limit of detection was found to be the lowest limit detected for the drug TDF at 3 µg/mL. Quantification of TDF was performed at 5 µg/mL due to the method's increased sensitivity. At the correct limit, this approach was found to have a high percentage recovery at low concentrations (Table I).

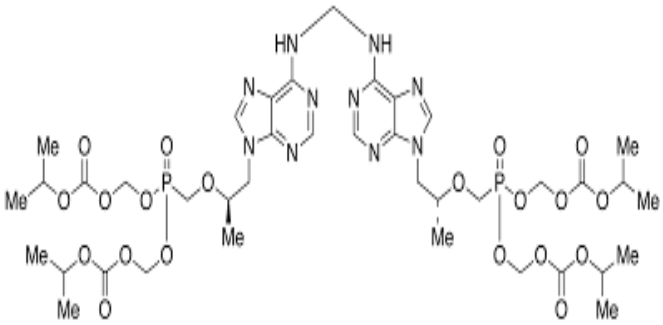
3.6 Robustness:

When the experimental conditions were modified, no major improvements in the chromatographic parameters were detected, indicating that the developed approach was found to be stable.

A novel liquid chromatography-tandem mass spectroscopy approach has been developed and validated, and it is simple, precise, effective, and reliable. The developed method can be used to calculate TDF in the API with success.

3.7 Confirmation of impurity:

Data collected from the UV, HPLC, LC-MS, IR and NMR showed that the synthesized TDF impurity probably be the Tenofovir disoproxil dimer.



Conclusion:

We can conclude from the above results that a simple, precise and novel method has been developed and validated. Synthesis and characterization of the TDF impurity gave tenofovir disoproxil dimer. The developed method can be successfully applied in various pharmaceutical method development.

The ADME/T (Absorption, Distribution, Metabolism, Excretion/Toxicity) properties of these compounds can be evaluated in a wet lab in the future, and experiments can be conducted.

Conflict of Interest:

The authors have no conflict of interest.

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AUTHORS

First Author – Rinchi Bora, Research Scholar, Department of Pharmaceutical Analysis, JSS College of Pharmacy, Ooty, Tamil nadu-643001, India.
Email: rinphd@jssuni.edu.in
ORCID: 0000-0002-0140-4125
Second Author – M.R. Jeyaprakash, M. Pharm, Ph.D
Department of Pharmaceutical Analysis
JSS College of Pharmacy
(A constituent college JSS Academy of Higher Education & Research) Ooty, The Nilgiris, Tamil Nadu, 643001, India
E-mails: jpvis7@jssuni.edu.in
Third Author – Samrat Debnath, Research Scholar, Department of Pharmaceutical Analysis, JSS College of Pharmacy, Ooty, Tamil nadu-643001, India
Email: Samratnath11@yahoo.com
Fourth Author – S. Jubie, M. Pharm, Ph.D
Department of Pharmaceutical Chemistry
JSS College of Pharmacy
(A constituent college JSS Academy of Higher Education & Research) Ooty, The Nilgiris, Tamil Nadu, 643001, India
E-mails: jubie@jssuni.edu.in
Correspondence Author – M.R. Jeyaprakash, M. Pharm, Ph.D
Department of Pharmaceutical Analysis
JSS College of Pharmacy
(A constituent college JSS Academy of Higher Education & Research) Ooty, The Nilgiris, Tamil Nadu, 643001, India
E-mails: jpvis7@jssuni.edu.in.
Mobile: +91 99523 35392
ORCID: 0000-0002-7411-302X