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FORMULATION AND CHARACTERIZATION OF TENOFOVIR DISOPROXIL FUMARATE POLYMERIC NANOPARTICLE

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1. ABSTRACT

There is a need to design alternative delivery systems to overcome the drawbacks of the current HIV/AIDS treatment therapy and improve patient compliance. The treatment of HIV/AIDS requires sustained release of the drug to reduce the frequency of the drugs as well as targeting to the tissues which act as latent HIV reservoirs, both of which can be successfully achieved bythe use of nanosystems for delivery of anti-retroviral drugs. Nanosystems are versatile drug delivery systems, with an ability to overcome physiologic barriers and to guide the drug to specific cells or intracellular compartments due to their small size, typically in the 10 1000 nm range. Nanosystems offer several advantages, such as the protection of drugs against degradation, targeting of drugs to specific sites, and tailoring the release kinetics to provide prolonged release of the drugs. Polymeric nanoparticles, solid lipid nanoparticles, liposomes, nanosuspensions and nanoemulsions have been reported to enhance the effective delivery of the drugs. Our research group has previously worked on development of particulate systems for Nelfinavir mesylate (NFV), a protease inhibitor.

2. KEYWORDS: Entrapment, nanoparticles, Anti-retroviral therapy, Formulation, polymer.

3. INTRODUCTION

4.1 Introduction to HIV

Disease outbreaks which turn into epidemic or pandemics have affectedthe world population

for decades. Ebola virus, H1N1, cholera, Zika virus, smallpox, polio and recently the Novel Coronavirus are a few diseases which have severely affected the global population. According to Morbidity and Mortality Weekly report of the CDC (Centre for disease control and prevention)[1], the first case of AIDS (Acquired Immunodeficiency Syndrome) caused by the Human Immunodeficiency Virus (HIV) was reported in 1981. Since then, HIV infections have grown to pandemic proportions with 74.9 million infected with HIV since then. 32.0 million people have died from AIDS-related illnesses since the start of the epidemic by the end of 2018, according to UNAIDS.

4.2 Current treatment regimen-Highly Active Anti-retroviral therapy (HAART)

Anti-retroviral therapy (ART) has aided in combating AIDS to a large extent by reducing the viral load, improving the life expectancy of patients, and reducing the transmission. Current regimen for AIDS is called HAART which is a combination of three drugs which target different enzymes in the HIV life cycle. According to the Joint United Nations programme on HIV/AIDS (UNAIDS), as of June 2019, 24.5 million people worldwide were accessing anti-retroviral therapy.[2] There are 32 anti-retroviral drugs approved by the USFDA which belong to different classes and on basis on clinical trials, it has been recommended that ART should be started as soon as feasible in an individual with HIV infection. However, there may be a need to change the regimen to maintain virologic suppression if the patient shows drug interactions or intolerability of the existing treatment. Change in regime may also be required in case of virologic failure where there is repeat detection of HIV RNA in the patient taking anti-retrovirals due to non-adherence of treatment or acquired drug resistance. ART is also prescribed as pre-exposure prophylaxis to patients who have a substantial risk of infecting HIV which generally consists of a two-drug regimen. Post exposure prophylaxis is recommended as soon as possible following exposure up to 72 hours which is generally a three-drug regimen.[5] Viral mutations has been the cause of ineffective ART and therefore, HIV guidelines[6] state that the patients with chronic infection should be tested for viral resistance to initial treatment. However, there are drawbacks of HAART which will be highlighted in the next section.

4.3 Drawbacks of current regimen

Even though HAART has been useful in controlling viral replication and decreasing mortality, there are certain disadvantages associated with the therapy. Some of them include:

- Anti-retroviral drugs are often poorly soluble in nature which leads to decrease in their oral

bioavailability.

- Shorter residence times of the drugs mean lesser concentration in the reservoir siteslike the lymphoid tissues, central nervous system, and lungs. Hence, prolonged duration with higher doses of drugs are needed to achieve optimal concentrations of the drug in the body which also leads to viral resistance.
- Patient compliance has also been low due to side effects and toxicity of the drugs associated with taking them for a longer time.

The high cost of HAART is another problem which increases the burden on the developing countries where the prevalence of the infection is the highest.

DRUG PROFILE

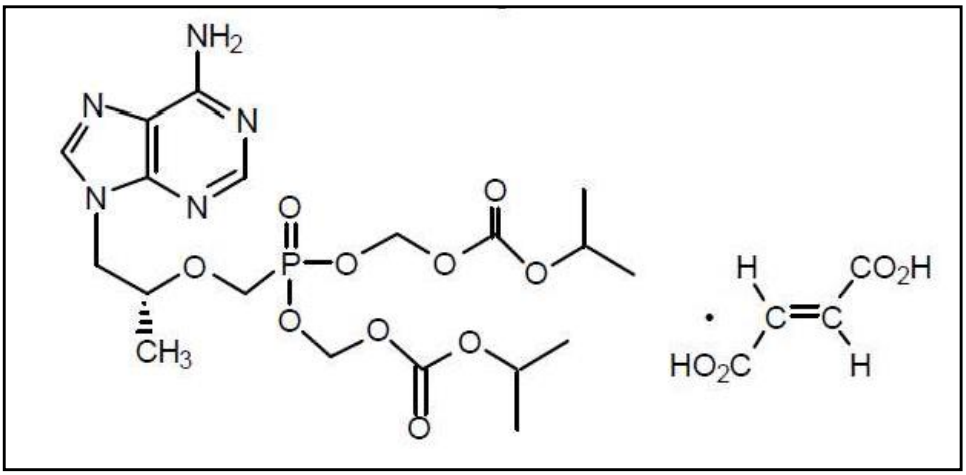


Fig.1.1. Structural Formula of Tenofovir Disoproxil Fumarate (TDF).

Chemical Name: [[(2R)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethyl-(propan-2-yloxycarbonyloxymethoxy) phosphoryl] oxymethyl propan-2-yl carbonate;(E)-but-2-enedioic acid.

Molecular Formula: $C_{19}H_{30}N_5O_{10}P \cdot C_4H_4O_4$

CAS Number: 202138-50

Molecular weight: 635.5 g/mol

Brands: Viread®

Description: White to off white crystalline powder

Melting Point: 113-115°C[166]

Therapeutic Category: Anti-retroviral agent

Solubility: TDF shows a solubility of 13.4mg/ml at 25 °C in water It is soluble in methanol, acetonitrile.

Mechanism of Action: On absorption in the gastrointestinal tract, the prodrug TDF is hydrolyzed to tenofovir. Tenofovir further undergoes phosphorylation to its active moiety tenofovir diphosphate. Tenofovir diphosphate inhibits the reverse transcriptase by competing with nucl-triphosphate and acting as a chain terminator, onincorporation in the DNA.

Pharmacokinetics

A) Absorption

TDF is a water-soluble prodrug of the active tenofovir. The oral bioavailability is approximately25%. The pharmacokinetics are dose proportional over the dosage range of 75-600mg and are not affected by repeat dosing.

B) Distribution

TDF is not highly bound to proteins with the binding for plasma and serum proteins being <1%and <7.2% over the concentration range 0.01-25mg/L. The binding to proteins is not concentration dependent. The volume of distribution is reported to be approximately 800mg/Lon intravenous administration of the drug to infected patients.

C) Metabolism

Tenofovir and tenofovir disoproxil fumarate are substrates of the CYP enzymes. Reports stated that no circulating metabolites were found in rats which indicated the lack of metabolism of tenofovir in the liver and intestinal homogenates.

D) Elimination

Renal excretion is the primary route of elimination of Tenofovir. Most of the drug is excreted unchanged through the urine by combination of tubular secretion and glomerular filtration. A study reported approximately 70-80% of the dose of tenofovir was excreted unchanged in theurine over 72hours after administration.

4. MATERIALS AND METHODS

➢ IDENTIFICATION OF TENOFOVIR DISOPROXIL FUMARATE

Materials

TDF was purchased from Carbosynth, USA. Potassium bromide and Methanol AR werepurchased from Himedia.

a) Description

The sample was visually examined for its appearance and color.

b) Infra-red (IR) spectroscopy

IR spectrum of pure TDF was recorded in the range from 4000cm^{-1} to 400cm^{-1} using KBrdisc method on FTIR spectrophotometer. (FT/IR-4100typeA).

c) Thermal analysis by Differential Scanning Calorimeter (DSC)

Differential scanning calorimetry was used for thermal analysis of drug using DSC 1 STARE system (Mettler Toledo). The drug was placed in aluminum pan and heated from 0°C to 125°C at a rate of 10°C /min. An empty pan was used as a reference pan.

d) Ultra-violet (UV) spectroscopy

The spectrophotometric measurements were carried out using an analytical double beam UV-Visible Spectrophotometer (Shimadzu UV-1900) with 1cm matched quartz cell. Stock solution of 1000 ug/ml was prepared in methanol AR. Further dilution of the stock solution were performed to obtain 10 ug/ml. The standard solution was scanned in the wavelength range of 200 nm to 800 nm against methanol as reference to determine the wavelength corresponding to maximum absorbance (λmax).

e) Crystallinity of EFV by X-Ray diffraction study (XRD)

X-Ray diffraction (XRD) studies were carried out to determine the crystallinity of Efavirenz. The diffraction patterns were recorded on Xpert Pro MPD (Pananalytical, The Netherlands) instrument with a copper anode, voltage 40 kV, current 30 mA, at a 1.5405 A. The detector used was Xcelerator Detector with diffracted beam monochromator. The studywas performed at Tata Institute of Fundamental Research, Mumbai.

➢ **Preparation and characterisation of TDF nanoparticles**

- **Materials**

Docusate sodium and Acetone AR grade was purchased from Himedia. Eudragit S100, EudragitEPO were obtained as gift samples from Evonik India. Signet chemical corporation kindly gifted the sample of cellulose acetate phthalate. Kolliphor 407 (Poloxamer 407) was gifted by BASF India.

✓ **Complexation of TDF with docusate sodium**

To enhance the entrapment of hydrophilic TDF, docusate sodium has been previously reported as the complexing agent .Three ratios of TDF: docusate sodium like 1:1, 1:1.5 and 1:2 were screened to provide maximum complexation efficiency. 10mg/ml TDF solution and

7mg/ml solution of docusate sodium were mixed in the aforementioned ratios and centrifuged (Minispin). The supernatant and pellets were analyzed using reverse phase HPLC to determine the complexation efficiency. The ratio with the maximum complexation efficiency was selectedin the preparation of the polymeric nanoparticles.

✓ **Screening of excipients and preparation of nanoparticles**

Various polymers like cellulose acetate phthalate (CAP), eudragit S100, eudragit EPO were screened along with Poloxamer 407 as the surfactant at different ratios for preparation of the nanoparticles. Based on earlier reports, acetone: Water in the ratio 1:1 was selected as the solvent system. The polymer and poloxamer were dissolved in 8ml acetone. TDF-docusate complex was dissolved in remaining amount of acetone and added to the above solution. The organic phase was added to the aqueous phase dropwise using a syringe on continuous stirring using a magnetic stirrer. Acetone was completely evaporated on stirring overnight. Optimized nanoparticles with CAP and Eudragit S100 as the polymers with Poloxamer 407 asthe stabilizers were labelled as C-NP and ES-NP respectively.

➢ **Characterisation of nanoparticles**

✓ **Particle size and polydispersity index (P.I.)**

Particle size and polydispersity index of C-NP and ES-NP were evaluated using particle size counter (N5 Submicron Particle Size Analyzer, Beckman Coulter, United States). It utilizes photon correlation spectroscopy technique and is based on the principles of dynamic light scattering. The formulations were appropriately diluted with freshly prepared MilliQ water (Type I) and particle size was determined at 25°C with 25 mW Helium Neon Laser (632.8nm) incident on the sample at an angle of 90° as the laser source. The data was analyzed using the PCS Software Version 3.02**.**

✓ **Entrapment efficiency (%EE)**

The entrapped TDF was separated from the free TDF in the nanoparticles by centrifugation. 0.5ml of the nanoparticles were placed in the ultra-centrifugal filters (Amicon®) which were centrifuged (Minispin) at 10,000rpm for 5 min. The filtrate was suitably diluted and analyzed for the TDF content by HPLC. Entrapment efficiency was calculated by indirectmethod using the following formula.

$$\%EE = \frac{\text{Total Drug-Drug in filtrate}}{\text{Total drug}} * 100$$

➢ **Anti-HIV activity testing using cell-based assays**

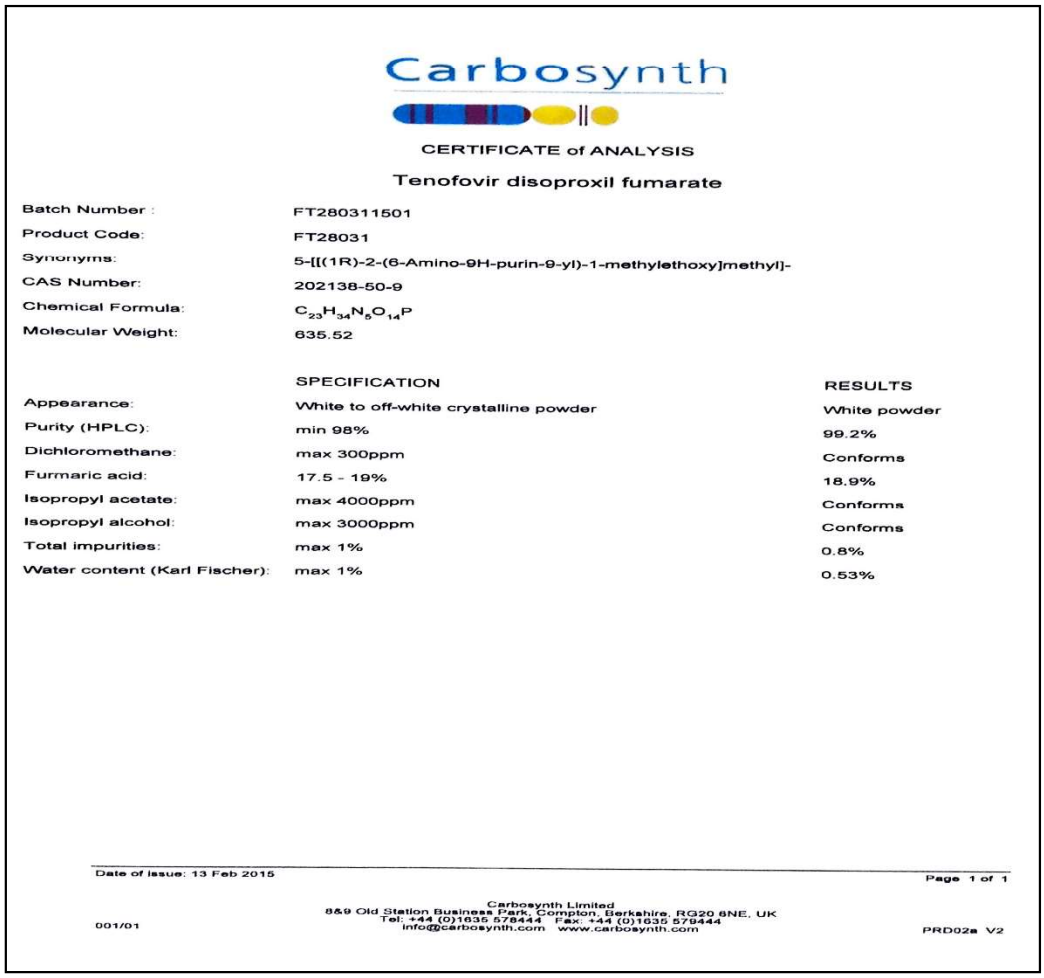
Anti-HIV testing was carried out National Aids Research Institute, Pune. Tenofovir disoproxil fumarate as the pure drug and nanoparticles C-NP, ES-NP were studied for their effect on TZM-bl cells infected with two viral strains, namely HIV-1 UG070 & VB28.

6. RESULTS AND DISCUSSION

Identification of Tenofovir disoproxil fumarate (TDF)

a. Description

Tenofovir disoproxil fumarate was a white crystalline powder. The certificate of analysis is shown in Fig.6.1.



Carbosynth

CERTIFICATE of ANALYSIS

Tenofovir disoproxil fumarate

Batch Number :	FT280311501
Product Code:	FT28031
Synonyms:	5-[[(1R)-2-(6-Amino-9H-purin-9-yl)-1-methylethoxy]methyl]-
CAS Number:	202138-50-9
Chemical Formula:	$C_{23}H_{34}N_5O_{14}P$
Molecular Weight:	635.52

	SPECIFICATION	RESULTS
Appearance:	White to off-white crystalline powder	White powder
Purity (HPLC):	min 98%	99.2%
Dichloromethane:	max 300ppm	Conforms
Furmaric acid:	17.5 - 19%	18.9%
Isopropyl acetate:	max 4000ppm	Conforms
Isopropyl alcohol:	max 3000ppm	Conforms
Total impurities:	max 1%	0.8%
Water content (Karl Fischer):	max 1%	0.53%

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001/01 PRD02a V2

Fig.6.1. Certificate of analysis of TDF.

b) Infrared Spectroscopy Study

The IR spectrum of TDF is shown in Fig.6.2. and is in accordance with the spectrum reported for TDF in IP 2010. The interpretation is shown in Table.6.1. shows that observed peaks were in accordance with the peaks which have been reported previously for TDF.

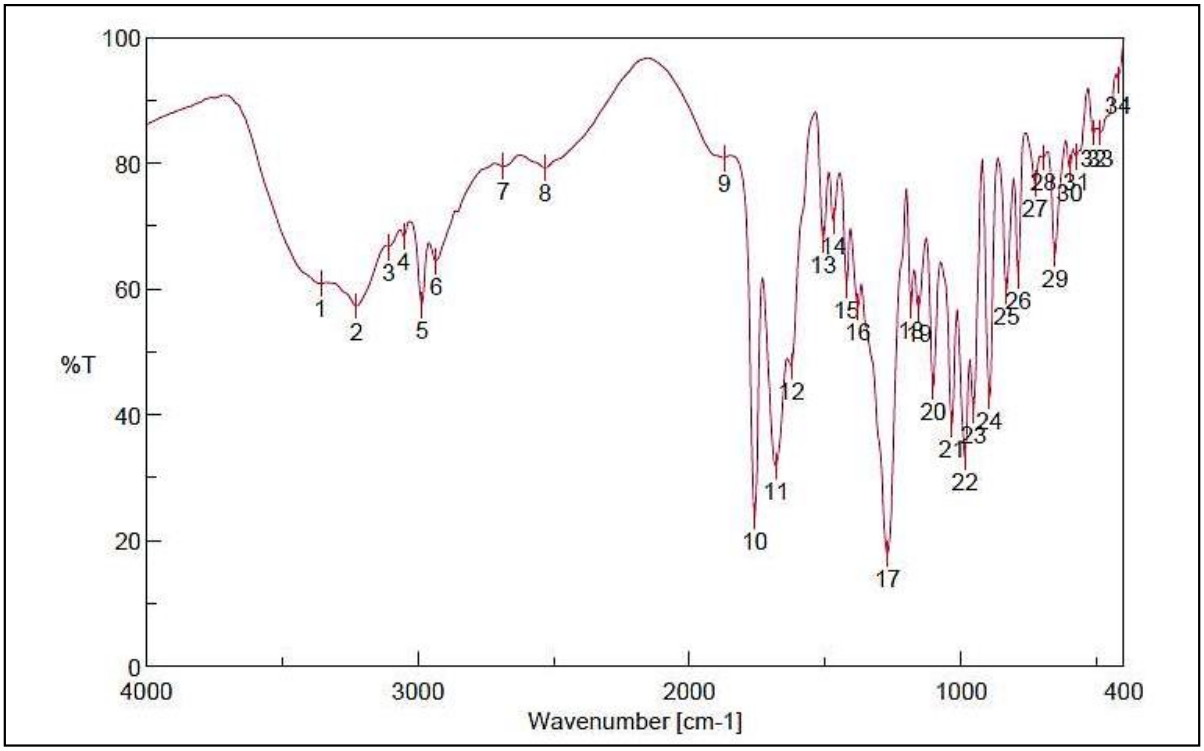


Fig.6.2. IR spectrum of Tenofovir Disoproxil fumarate

Table 6.1. Reported and observed IR peaks of TDF.

Reported Peaks (cm^{-1})	Observed peaks (cm^{-1})	Functional Groups
3061	3051.8	=C H stretch alkenes
2987	2986	C H stretch alkanes
2686	2686	H C=O: C H stretch aldehydes
1761	1759	C=O stretch
1680	1624	N H bend 1° amines
1465	1421	C C stretch (in ring) aromatics
1269	1270	C N stretch aromatic amines
1035	1033	C O stretch alcohols, carboxylicacids, esters, ethers
987	953	O H bend carboxylic acids
792	788	N H wag 1°, 2° amines
650	696	N H wag 1°, 2° amines

b) Differential Scanning Calorimetry

Fig.6.3. shows the DSC thermogram of TDF. A sharp endothermic peak was observed at 114°C which corresponds to the melting point of TDF and is in accordance with the reportedliterature.

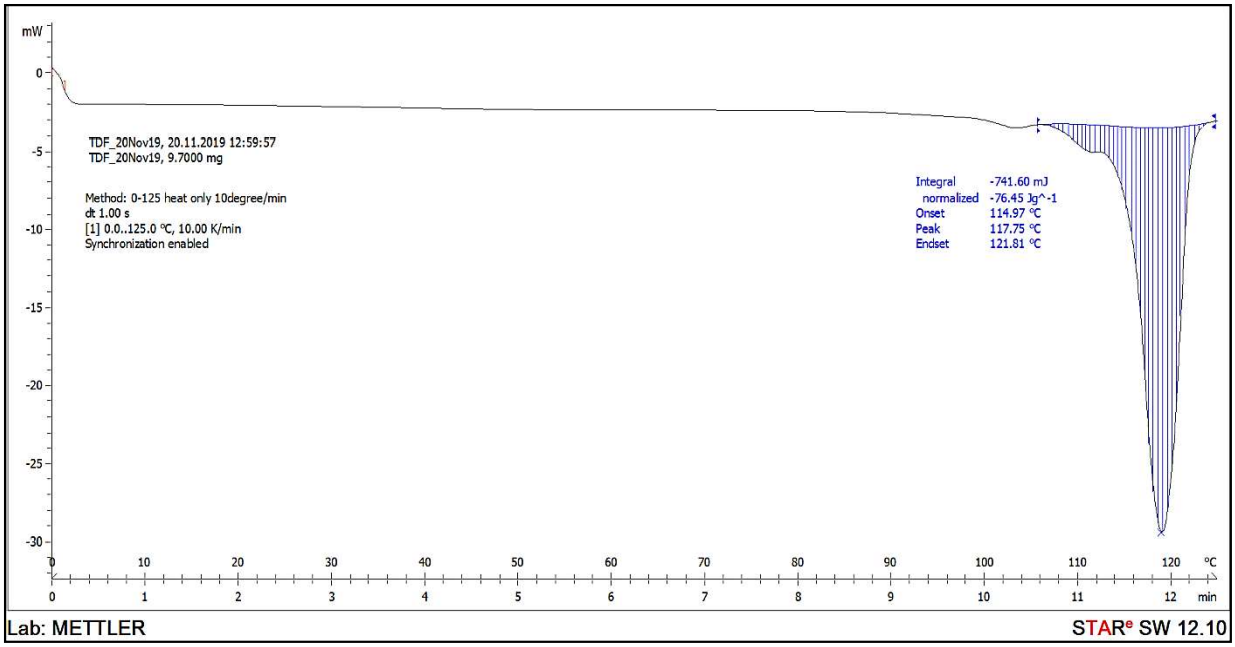


Fig. 6.3. DSC thermogram of TDF.

b) Ultraviolet spectral study

A stock solution of 10ppm TDF in methanol was scanned using the UV spectrophotometer(Shimadzu, UV-1900) in the range of 200-400nm. Fig.7.4. shows the UV scan of TDF with a max of 260nm which is in accordance with reports in the literature.

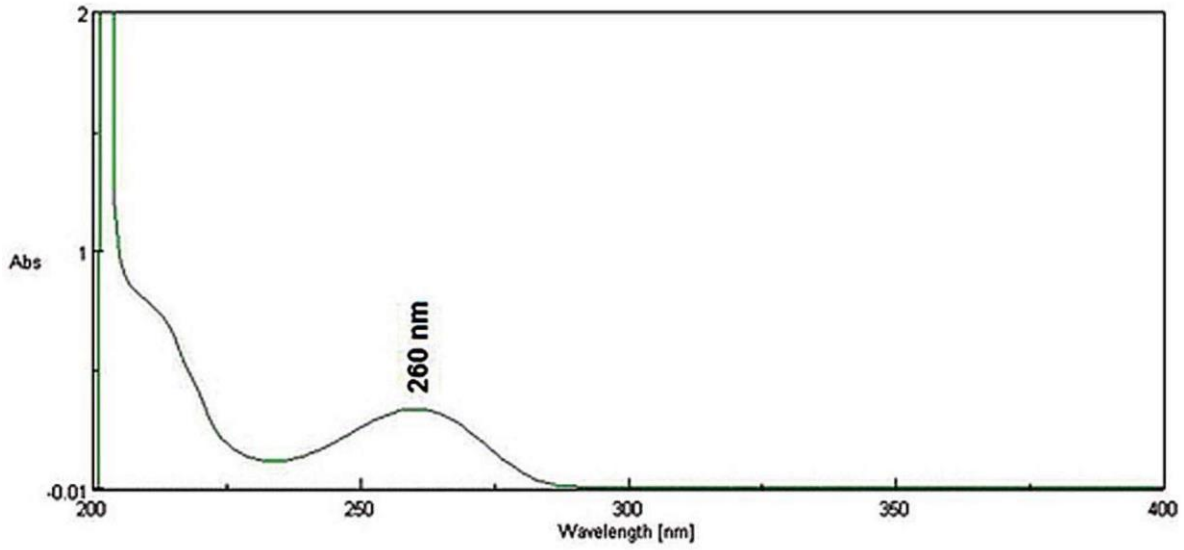


Fig. 6.4. UV spectrum of TDF in methanol.

b) X-Ray diffraction study

Fig.7.5. shows the XRD pattern of TDF. The diffraction patterns of TDF show two distinct peaks at 2 of 19.8 and 25℃ which are indicative of the crystalline nature of the drug

and are in line with the reports.

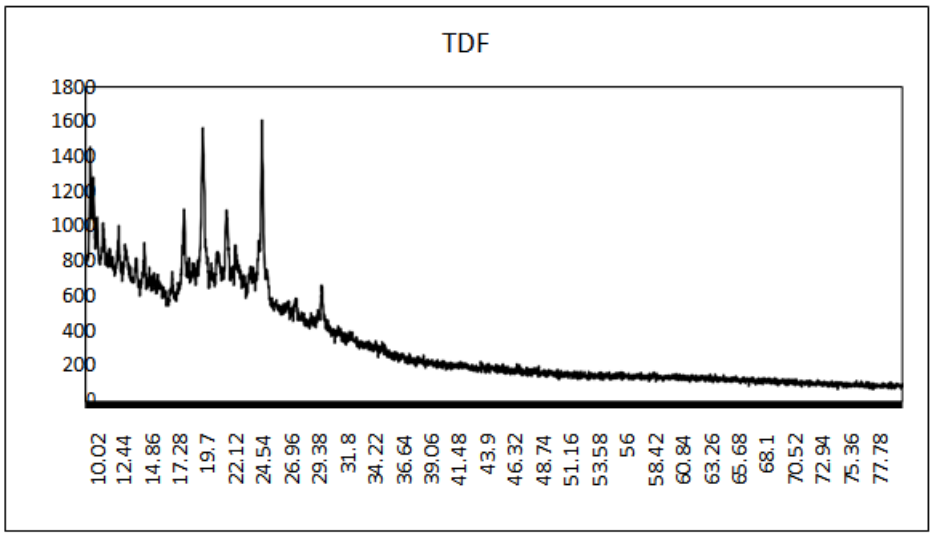


Fig.6.5. XRD pattern of TDF.

➢ **Analytical method development by HPLC**

The current method developed for the analysis of TDF resulted in a well resolved peak with the retention time of 7.186±0.007 min and a symmetrical peak shape (tailing factor 1.2). Fig.7.6. shows the chromatogram of 10ppm solution of TDF.

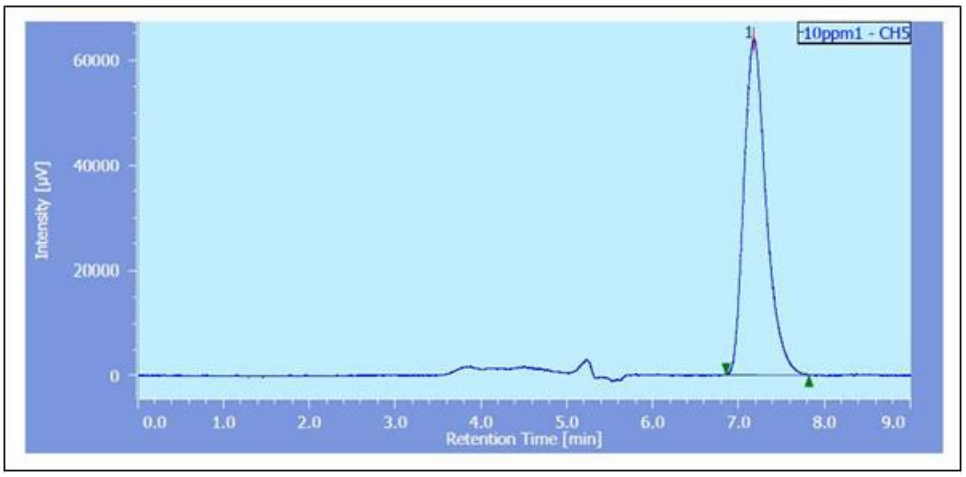


Fig.6.6. Chromatogram of 10ppm TDF solution.

➢ **Preparation and characterisation of nanoparticles**

✓ **Complexation of TDF with Docusate sodium**

✓ Table.6.9. shows the amount of TDF and docusate sodium used for complexation in accordancewith the molar calculations.

Table.6.9. Amount of TDF and docusate sodium used for complexation.

Ratio	TDF (mg/ml)	Docusate (mg/ml)
1:1	10	7
1:1.5	10	10.5
1:2	10	20

Screening of excipients and preparation of nanoparticles

✓ CAP-Cellulose acetate phthalate, P407- Poloxamer 407, ES100- Eudragit S100.

Table 6.10. Initial trials of TDF nanoparticles.

Polymer	Surfactant	Polymer load (mg)	Surfactant load (mg)	Particle size(nm)	Polydispersity index	%Entrapmentefficiency
CAP	P407	100	200	183.7±1.97	0.049±0.034	32.2
CAP	P407	150	400	228.8±2.41	0.111±0.076	50.1
CAP	P407	50	200	147.0±0.89	0.182±0.040	12.4
CAP	P407	150	200	230.9±3.84	0.326±0.029	37
CAP	P407	150	300	204.0±0.007	0.094±0.010	50
ES100	P407	200	200	117.4±0.96	0.745±0.016	NA
ES100	P407	300	300	42.0±0.62	0.499±0.010	NA
ES100	P407	100	100	133.5±0.91	0.407±0.030	33
ES100	P407	150	150	150.3±0.59	0.443±0.006	5

Table 6.11. Effect of complexation on %EE of nanoparticles.

Polymer	Surfactant	Polymerload (mg)	Surfactantload (mg)	Particle size (nm)	Polydispersityindex	%EE	TDF: Docusate Complex
ES100	**P407**	**150**	**150**	**167.7±3.57**	**0.460±0.002**	**62.75**	**1:1**
ES100	P407	150	150	151.0±0.12	0.540±0.026	50.2	1:1.5
ES100	P407	150	150	136.7±0.19	0.524±0.026	54.3	1:2
CAP	**P407**	**150**	**300**	**264.6±14.37**	**0.332±0.099**	**80.3**	**1:1**
CAP	P407	150	300	246.9±2.31	0.037±0.030	73.1	1:1.5
CAP	P407	150	300	222.9±0.69	0.083±0.031	77.5	1:2

➢ **Characterization of nanoparticles**

✓ **Particle size distribution**

Table 7.13. Particle size distribution of polymeric nanoparticles.

Formulation	Particle size (nm)	Polydispersity index
C-NP	264.6±14.37	0.332±0.099
ES-NP	167.7±3.57	0.460±0.002
Conditions of Measurement: 1. Temperature: 25°C 2. Angle: 90° 3. Diluent: Water 4. Diluent Viscosity/ RI: 0.890 cP / 1.333		

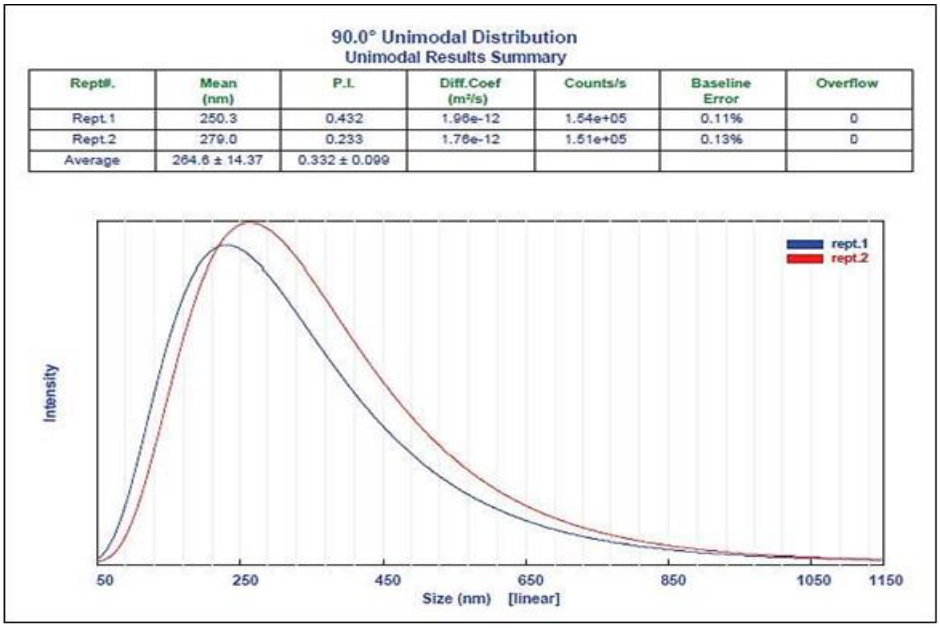
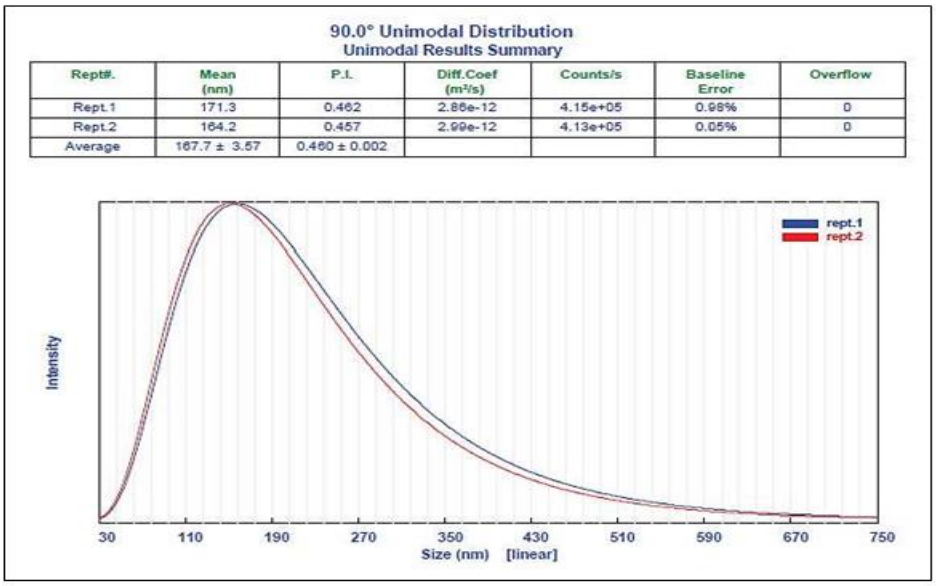


Fig. Particle size distribution of C-NP.



- **Entrapment efficiency**

Table. Entrapment efficiency of TDF nanoparticles.

Formulation	Ratio of TDF: Docusate sodium	% Entrapment efficiency
C-NP	-	50±0.57
	1:1	80.3±0.12
	1:1.5	73.1±1.00
	1:2	77.5±0.73
ES-NP	-	5±0.3
	1:1	62.75±0.89
	1:1.5	50.2±0.65
	1:2	54.6±1.01

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