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Original Article

Development and validation of a stability-indicating lc method for the determination of tenofovir disoproxil fumarate in pharmaceutical formulation

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Abstract

The present study describes the degradation of tenoforvir disoproxil fumarate (teno) under different prescribed stress conditions (hydrolysis, oxidation, dry and wet heat and photolysis) following the International Conference on Harmonization and application of a specific and selective stability-indicating reversed-phase high–performance liquid chromatography (HPLC) assay. Separation of drug and degradation products was successfully achieved on C_{18} analytical column using methanol: water (60:40, v/v) at a flow rate of 1.0 ml/min and detection at 260 nm, the mass balance was found to be close to 100.4%. The developed HPLC method was validated with respect to linearity, accuracy, precision, robustness, and accuracy.

Keywords: HPLC, forced degradation, validation, stress study, tenoforvir disoproxil fumarate

1. Introduction

Tenofovir is intracellularly converted to diphosphate. This diphosphate halts the DNA synthesis of HIV through competitive inhibition of reverse transcriptase and incorporation into viral DNA. It also inhibits hepatitis B virus polymerase, resulting in inhibition of viral replication. It is used in the treatment of HIV infection and chronic hepatitis B infection. Tenofovir disoproxil fumarate (short: teno) is a fumaric acid salt of the bisisopropoxycarbonyloxymethyl ester derivative of tenofovir. Chemically it is 9-[(R)-2-[[(isopropoxcarbonyl)-oxy]methoxy]phosphiny] methoxy]propyl] adeninefumarate (Figure 1). It is not official in any of the pharmacopoeias. This is listed in the Merck Index and Martindale: The complete drug reference can be found at a Budawari, *et al. (*2001) and Sweetman, *et al. (*2005).

Literature review reveals that several methods have been reported for the estimation of teno in tablets (Patel *et al.,* 2009; Raju and Begum *et al.,* 2009; Karunakaran *et al.,* 2010; Behera *et al.,* 2011), using high-performance liquid chromatographic (HPLC) methods (Jullien *et al.,* 2003; Sentenac *et al.,* 2003; Rezk *et al.,* 2005; Kandagal *et al.,* 2008; Seshachalam *et al.,* 2008), LC-MS (Bezy *et al.,* 2005; Delahunty *et al.,* 2006; King *et al.,* 2006; Barkil *et al.,* 2007; Massaki *et al.,* 2007; Delahunty *et al.,* 2009), and high-performance thin layer liquid chromatographic methods (Joshi, *et al.* 2006).

HPLC is a versatile technique, and HPLC with UV detection is often preferred in ordinary laboratories and quality controlled laboratories because of its wide suitability and availability. So far, to our present knowledge, no stability-indicating LC assay method for the determination of teno is available in the literature. It was felt necessary to develop a

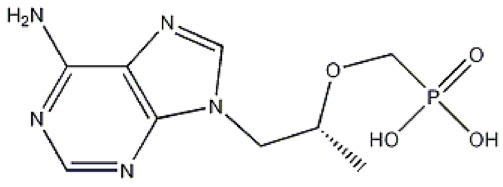

Figure 1. Chemical structure of tenofovir disoproxil fumarate.

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stability indicating LC method for the determination of teno as bulk drug and pharmaceutical dosage form and separate the drugs from the degradation products under the ICH (International Conference on Harmonization) suggested conditions.

The aim of the present study was to establish the inherent stability of tenofovir disoproxil fumarate through stress studies under a variety of ICH recommended test conditions (International Conference on Harmonization, 1996; Singh and Bakshi *et al.,* 2000; International Conference on Harmonization, 2003) and to develop a stability-indicating assay (Carstensen *et al.,* 2000; Singh and Bakshi *et al.,* 2002; Singh *et al.,* 2006).

2. Experimental

2.1 Chemical and reagents

Pharmaceutical grade teno working standard was obtained as generous gifts from Ranbaxy Pvt. Ltd. Indore, India. Commercially available Tentide tablets (300 mg) [T-I] were purchased from Ranbaxy Pvt. Ltd. India and Tavin (300 mg) [T-II] from Emcure pharmaceuticals. All chemicals and reagents were of HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

2.2 Instrumentation

2.2.1 Stress study

High precision heating mantel (Narang Scientific Works, New Delhi, India) capable of controlling the temperature with in ±1°C was used for generating hydrolytic degradation products. The thermal degradation study was performed using a high precision hot air oven (Kumar Scientific Works, Pune, India) capable of controlling the temperature with in ±2°C. Photo-degradation was carried out in a photostability chamber (Thermolab, Scientific Equipment Pvt Ltd.) equipped with lighting system to comply with ICH guideline for photostability condition with white fluorescent light exposure for 1.2 milion lux hours and integrated near ultra violet energy exposure of 200 watts hours/sq mts (Option 2 of the ICH guideline Q1B). At any given time, UV energy and visible illumination were tested using a calibrated lux meter (Lutron, LX-101A).

2.2.2 LC–UV analyses

The LC system consisted of a pump (model Jasco PU1580, intelligent LC pump) with auto injecting facility (AS-1555 sampler) programmed at 20 µl capacity per injection. The detector consisted of a UV–vis (Jasco UV 1575) model operated at a wavelength of 260 nm (Figure 2). The software used was Jasco borwin version 1.5, LC-Net II/ADC system. The column used was HiQ Sil C18HS (250 mm×4.6 mm, 5.0 µm) Kya Technologies Corporation, Japan.

2.3 Preparation of standard stock solutions

Standard stock solutions of teno was prepared in acetonitrile of concentration 1 mg/ml. Working standard solutions were prepared by serial dilution of the stock solution with the mobile phase.

2.4 General assay procedure

2.4.1 Preparation of the calibration graph

Working standard solutions containing 4.0–20.0 µg/ml of teno were prepared by serial dilution of aliquots of the stock solution and triplicate injections were made of each solution and the peak areas of teno were plotted against the corresponding concentration (µg/ml) to obtain the calibration graph. The corresponding regression equation was derived.

2.4.2 Analysis of bulk substance

The method mentioned above was applied for the determination of the purity of teno raw material. The percentage recoveries were calculated by referring to the calibration graph previously prepared or applying the regression equation.

2.4.3 Procedure for stress testing

A stock solution containing 100 mg teno in 100 ml acetonitrile was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating ability and specificity of the proposed method. In all degradation studies, the average peak area of teno (10 µg/ml) after application of six replicates was obtained.

Hydrolysis: 5 ml of a standard stock solution (1 mg/ml) was mixed with 5 ml 0.01 M HCl at room temperature. The alkaline hydrolysis was carried out by mixing a standard stock solution 5 ml (1 mg/ml) with 0.01 M NaOH (5 ml) kept at room temperature. The solution was then neutralized with 0.01M NaOH and 0.01 M HCl for the acidic and alkaline degradation, respectively

Oxidation: For the purpose of oxidation studies a mixture of 5 ml standard stock solution and 5 ml hydrogen

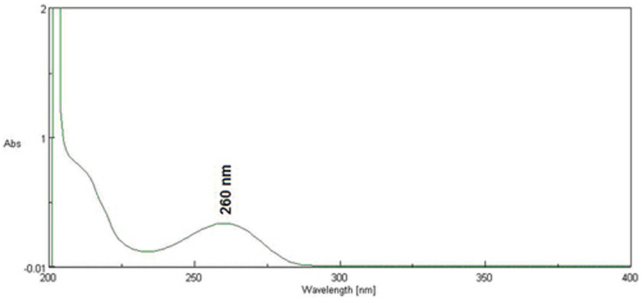

Figure 2. UV absorption spectrum of tenofovir (λ_{max}= 260).

peroxide (0.3%, v/v) was kept at room temperature and then heated in a boiling water bath for 10 min to completely remove the excess hydrogen peroxide.

Dry heat degradation: 10 mg standard drug as powder was placed in an oven at 50°C for two months to study the dry heat degradation.

Photochemical degradation: Photo degradation studies were carried out according to Option 2 of Q1B in the ICH guidelines. The stock solution (1 mg/ml) as well as the solid drug was exposed to light for an overall illumination of 1.2 million lux/hr and an integrated near ultraviolet energy of 200 W hm^{-2} for 8 hrs.

Neutral hydrolysis: To study the degradation behavior of drug in neutral conditions, 5 ml of a standard stock solution (1 mg/ml) was mixed with 5 ml double distilled water and heated at 80°C for 5 days, and subsequently for 10 days.

2.4.4 Analysis of dosage forms

For the analysis of tablets, 20 tablets of each batch T-I and T-II were weighed and finely ground in a mortar. For T-I and T-II, the portion equivalent to 300 mg of teno was transferred in a 50 ml volumetric flask, 35 ml of acetonitrile was then added, and sonication was done for 45 min with swirling. After sonication, the volume was made up to the mark with the acetonitrile and mixed well. The solution was filtered through Whatman filter paper 41. For both T-I and T-II, six determinations were performed.

2.5 Optimization of the stability-indicating LC method

The LC procedure was optimized with the objective of developing a stability indicating assay. After performing photo degradation of the drug, dilutions were injected and chromatographed with different mobile phases. Initially mobile phases containing various proportions of methanol: water were tested. Resolution of the drug peak was good but peaks of the degradation products were not resolved satisfactorily. To improve the resolution of the peaks of the degradation products methanol: water (60:40, v/v) was evaluated as mobile phase which enabled good resolution of the drug from its degradation products. This mobile phase was used at a flow rate of 1.0 ml/min and detection at 260 nm gave acceptable retention time (t_R), theoretical plate number, and resolution of the drug and its degradation products

2.6 Analytical method validation

The developed chromatographic method was validated for linearity, precision, accuracy, sensitivity, robustness and system suitability.

2.6.1 Linearity and range

Linearity of the method was studied by injecting the drug solution prepared in the mobile phase in the concentration range of 4–20 µg/ml, injected six times into the LC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

2.6.2 Precision

Precision of the method was evaluated by analysis of three independent sample preparations. The determination of RSD (%) value obtained from three assay values. These studies were repeated on three different days to determine inter-day variation. Precision studies were carried at a drug concentration level of 4, 12, and 20 µg/ml.

2.6.3 Sensitivity

Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were calculated as 3.3 and 10 ó/S, respectively.

2.6.4 Robustness and system suitability

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ±0.1 ml/min), mobile phase composition (methanol ±5 ml).These chromatographic variations were evaluated for resolution between teno and photo degradation products.

2.6.5 System suitability criteria

The system suitability was assessed by five replicate analyses of the drugs at a concentration of 4 µg/ml of teno.

2.6.6 Specificity

The specificity of the LC method was determined by the complete separation of teno in presence of its degradation products along with other parameters like retention time, capacity factor, tailing, asymmetrical factor, and others. Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the teno and degradation products. For determining selectivity of the method, a powder blend of typical tablet excipients containing lactose monohydrate, mannitol, maize starch, povidone K30, citric acid anhydrous granular, sodium citrate, natural lemon and lime flavour, acesulfame potassium and magnesium stearate was prepared and analyzed. All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks.

2.6.7 Accuracy

Accuracy of the method was tested by applying the method to drug sample to which known amounts of teno

standard powder corresponding to 80, 100, and 120% of label claim had been added (standard addition method), mixed and the powder was extracted and analyzed by running chromatograms in optimized mobile phase. These mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recovery (%) was calculated.

2.6.8 Analysis of marketed formulation

The contents of drug in tablets were determined by the proposed method using the calibration curve.

2.6.9 Solution stability and mobile phase stability

The solution stability of teno was carried out by leaving the test solution in tightly capped volumetric flasks at room temperature for 24 hrs and assayed at 6 hrs interval, against the freshly prepared standard solution. The mobile phase stability was carried out by assaying the freshly prepared standard solution at 24 hrs interval up to 48 hrs. The %RSD of assay of teno was calculated for the study period during mobile phase and solution stability experiments.

3. Results and Discussion

3.1 Method development and optimization

Initially, mobile phases containing various proportions of methanol:water were tested. Sufficient separation between drug and degradation products was observed using methanol:water (60:40, v/v) gave acceptable retention time (t_R 12.09 min) (Figure 3).

3.2 Detection of degradation products by HPLC

3.2.1 Hydrolysis

Acid and alkaline degradation of teno was performed in 1:1 acetonitrile–0.01 M HCl and NaOH. teno was highly susceptible to attack by HCl and NaOH. Complete degradation occurred immediately after addition of HCl and NaOH at room temperature.

3.2.2 Oxidation

The drug was found to be unstable to oxidative degradation. In 1:1 acetonitrile– 0.3% H_2O_2 complete degradation occurs immediately at room temperature.

3.2.3 Dry and wet heat degradation product

There was no significant degradation of solid teno on exposure to dry heat at 50°C for two months, which indicated that drug was stable against thermal stress.

3.2.4 Photolysis

Teno was degraded in photochemical degradation after exposing drug to a combination of white fluorescent and integrated near ultra violet energy at 1.2 million lux/hr and 200 watts hours/sq mts, respectively for 8 hrs forming three major degradation products at 1.14 and 2.01 min (Figure 4).

3.2.5 Neutral degradation

Teno under neutral hydrolysis did not give rise to the presence of degradants as the peak area remained constant which indicated drug stability under the conditions investigated.

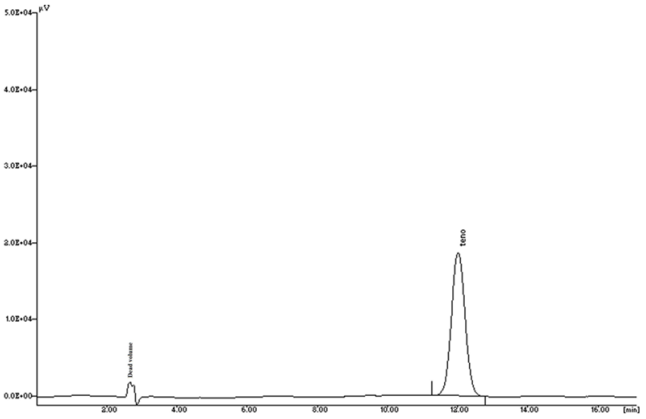

Figure 3. Chromatogram of tenoforvir disoproxil fumarate (20 mg/ml), t_R : 12.01 min; measured at 260 nm, mobile phase: methanol:water (60:40 v/v).

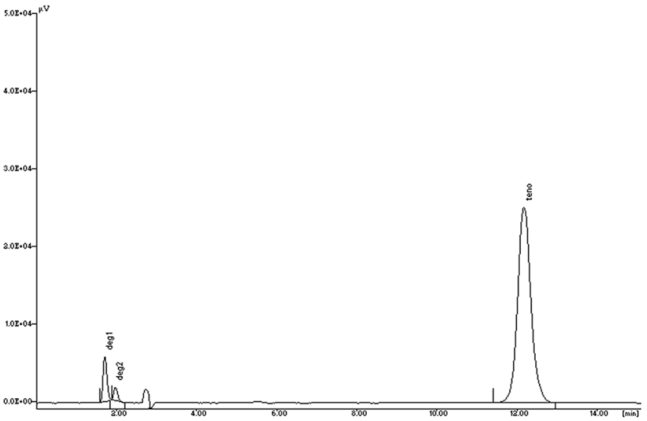

Figure 4. Chromatogram of photo degradation of tenofovir (10 µg/ml); peak 1 (degraded) (t_R: 1.14 min), peak 2 (degraded) (t_R: 2.01 min), peak 3 (tenofovir disoproxil fumarate) (t_R: 12.16 min).

3.3 Validation

3.3.1 Linearity

Good linearity was observed in the concentration range of 4–20 µg/ml of teno (Figure 5). The data was subjected to statistical analysis using a linear regression model; the result shows that within the concentration range mentioned above, there was an excellent correlation between peak area and concentration of each drug (Table 1).

3.3.2 Precision

The developed method was found to be precise, with %RSD values for repeatability 0.09% and 0.46% for interday precision, as recommended by ICH guidelines.

3.3.3 LOD and LOQ

The LOD and LOQ values were found to be 0.01 and 0.03 µg/ml.

3.3.4 Specificity

Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the drugs. These results demonstrate that there was no interference from other materials in the tablet formulation; therefore, confirm the specificity of the method (Figure 6).

3.3.5 System suitability

System suitability parameters such as the number of theoretical plates, HETP and peak tailing were determined. The results obtained are shown in Table 2.

3.3.6 Robustness of the method

Each factor selected was changed at three levels (-1, 0, and 1). One factor at a time was changed to determine the effect. Thus, replicate injections (n=6) of mixed standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Insignificant variability in retention time, capacity factor and U.S.P. tailing factor were observed (Table 3).

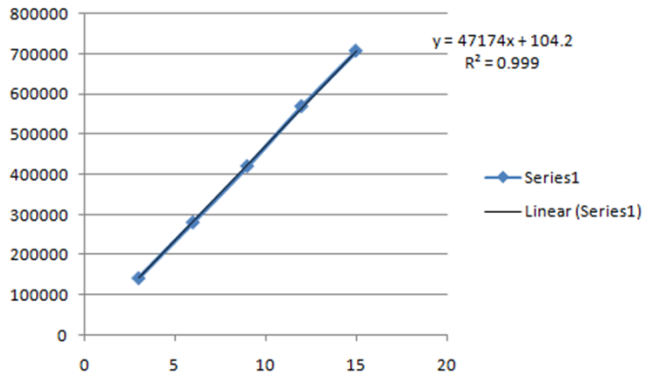

Figure 5. Calibration curve for tenofovir.

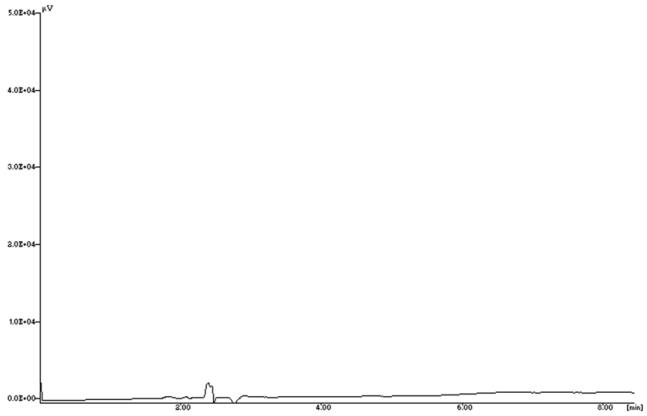

Figure 6. Representative chromatogram obtained for the placebo.

Table 1. Linear regression data for the calibration curves[a].

Compound	Linearity (µg/ml)	y = A + Bx		r^2
		A	B	
teno	4-20	104.2	4717	0.9999

[a] n = 6; r^2, coefficient of correlation

Table 2. Statistical analysis of parameters required for system suitability testing of the proposed HPLC method (n = 3 determinations).

Parameters	Teno
N	5156.2120
t_R	12.16
T	1.0374
% R.S.D.	0.04

Table 3. Robustness testing[a].

Chromatographic Factors[a]	Level	t_R[b]	k[c]	T[d]
Flow rate (ml/min)	0.9	12.74	2.26	1.18
	1.0	12.16	2.28	1.03
	1.2	12.02	2.43	1.00
% of methanol (v/v)	55	12.98	2.45	1.29
	60	12.16	2.28	1.03
	65	12.03	1.22	1.01

[a] n = 6, t_R = Retention time in minutes; T = USP tailing factor, k[c] = capacity factor

Table 4. Recovery studies[a].

Label claim	Amount of drug added (%)	Total amount of drug present (µg/ml)	Amount found (µg/ml)	% Recovery
		T-I		
300 mg	80	10.8	10.76	99.62
	100	12	11.92	99.33
	120	13.2	13.13	99.46
		T-II		
300 mg	80	10.8	10.71	99.16
	100	12	11.96	99.66
	120	13.2	13.17	99.77

[a] n = 6

3.3.7 Solution and mobile phase stability studies

No additional peak was found in the chromatogram. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable up to 48 hrs for assay and related substances analysis.

3.3.8 Recovery studies

Good recoveries of the teno were obtained at various added concentrations for T-I and T-II as shown in Table 4.

3.3.9 Analysis of a commercial formulation

Experimental results of the amount of teno in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients, which are normally present in tablets. Two different brands of fixed dose combination tablets were analyzed using the proposed procedures (Table 5).

Finally, a summary of the validation parameters are listed in Table 6 and of the stress study in Table 7.

Table 5. Applicability of the HPLC method for the analysis of the pharmaceutical formulations.

Label claim (mg)	Sample	Drug Content (%)	% R.S.D.
300	T-I	99.90	0.07
	T-II	100.35	0.10

Table 6. Summary of validation parameters.

Parameter	Teno
Linearity range (µg/ml)	4-120
Correlation coefficient	0.999±0.81
Limit of detection (µg/ml)	0.01
Limit of quantitation (µg/ml)	0.03
Recovery (n=6)	
T-I	99.47
T-II	99.53
Precision (% R.S.D.)	
Repeatability	0.09
Inter day	0.46
Robustness	Robust
Specificity	Specific

Table 7. Summary of the stress study.

Condition	Time (hrs)	Assay of active substance (%)	Mass balance (% assay + % sum of impurities + sum of all degradents)	Degradation (%)	t_R of major degradation product (min)
Exposed to a combination of white fluorescent and integrated near ultra violet energy at 1.2 mil lux hours and 200 watts hours/sq mts, respectively	8	91.93	100.29	5.96, 1.96	1.14, 2.01

4. Conclusion

This study showed that tenofovir disoproxil fumarate was found to be unstable under acidic, alkaline and oxidative conditions as it degraded completely, but it was found that it is labile to photolysis and that complete separation of degradants was carried out using an isocratic stability-indicating HPLC method. Tenofovir disoproxil fumarate was observed to be stable when exposed to neutral condition, wet and dry heat. The developed HPLC method proved to be simple, accurate, precise and specific. Hence, it is recommended for industrial analysis of drug and degradation products obtained from stability procedures.

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