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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE DETERMINATION OF TENOFOVIR DISOPROXIL IN BULK AND IN PHARMACEUTICAL FORMULATION

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ABSTRACT

Tenofovir disoproxil (TD) is a prodrug of tenofovir. It is a novel antiviral drug, which is highly efficient in the treatment of human hepatitis B virus (HBV) and HIV. A simple, selective, linear, accurate and precise RP-HPLC method was developed and validated for routine analysis of tenofovir disoproxil in bulk and in pharmaceutical formulation. Isocratic elution at a flow rate of 1.0 mL/min was employed on a Thermo Hypersil HyPURITY C18 reversed-phase column (150 mm × 2.1 mm i.d., 5 µm) at ambient temperature. The mobile phase consisted of acetonitrile and 0.05 mM phosphate buffer pH 6.0 (50 : 50, v/v). The UV detection wavelength was 260 nm and 20 µL of sample was injected. The retention time for tenofovir disoproxil is 4.45 min. Determining sensitivity, accuracy, precision, robustness, stability, specificity, selectivity, and system suitability parameters validated the proposed method.

Keywords: Tenofovir disoproxil, RP-HPLC, Validation.

INTRODUCTION

Chemically, tenofovir disoproxil (TD) is (bis (POC) PMPA), known as 9-((R)-2-((bis (((isopropoxycarbonyl) oxy] methoxy) phosphinyl) methoxy) propyl) adenine[1,2] (Fig.1). Tenofovir disoproxil (TD) is a prodrug of tenofovir. It is a novel antiviral drug, which is highly efficient in the treatment of human hepatitis B virus (HBV) and HIV. Tenofovir is an acyclic nucleoside analogue of adenosine monophosphate. *In vivo*, it is converted to an acyclic nucleoside phosphate, which is an analogue of adenosine 5-monophosphate called tenofovir diphosphate. Tenofovir is phosphorylated to the active metabolite, tenofovir diphosphate, by cellular kinases; tenofovir diphosphate inhibits (HBV)

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DNA polymerases (reverse transcriptase) by competing with the natural substrate deoxyadenosine triphosphate and by causing DNA chain termination after its incorporation into viral DNA[3]. Nucleotide reverse transcriptase inhibitors and nucleoside reverse transcriptase inhibitors (NRTIs) work in the same manner to block HIV replication. This is accomplished by competing with the natural DNA substrates to inhibit reverse transcriptase and subsequently decreasing or preventing HIV replication in infected cells[4]. It is used in combination with other retroviral, for the treatment of HIV infection. It is given orally in a dose of 300 mg of disoproxil fumarate ester, once daily with food. The base has been given intravenously, with evidence of active viral replication and either evidence of persistent elevations in serum aminotransferases (ALT or AST) or histologically active disease.

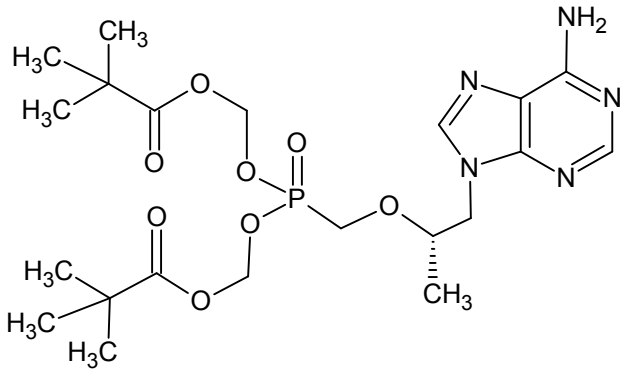

Fig. 1: Chemical structure of tenofovir disoproxil (TD)

Tenofovir disoproxil fumarate is absorbed rapidly and extensively after oral administration, and approximate oral bioavailability of tenofovir from tenofovir disoproxil fumarate is 59%. *In vitro* binding of tenofovir to human plasma or human serum proteins is 4% over the tenofovir concentration range of 0.1 to 25 mg/mL. Metabolism involves the rapid conversion of tenofovir disoproxil fumarate to tenofovir, 45% of the dose[5]. tenofovir is renally excreted by a combination of glomerular filteration and active tubular secretion[6]. Since tenofovir disiproxil is widely used in the antiviral therapy, it is important to develop and validate analytical methods for its determination in pharmaceutical dosage form.

Hitherto, there are few analytical methods reported for estimation of tenofovir disoproxil; sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography[7]; Development and validation of a sensitive LC-MS/MS method for the determination of TD in human plasma[8]. These methods are complicated, costly and time consuming rather than a simple RP-HPLC method. So it is unsuitable to use

these highly sensitive methods for the routine quantitative assay of TD tablets, where the content of active pharmaceutical ingredient is high in formulation.

The aim of the present work was to develop and validate a simple, fast and reliable isocratic RP-HPLC method with UV detection for the estimation of TD in bulk and in tablets. The important features and novelty of the proposed method included simple sample treatment with sonication of small amount of powder sample at ambient temperature, dilution; short elution time (less than 5 min), short analysis time (less than 30 min); good precision (R.S.D. less than 5%) and high recovery (greater than 95%). Confirmation of the applicability of the developed method validated according to the International Conference on Harmonization (ICH), for determination of TD in tablets has been also performed.

EXPERIMENTAL

Material and methods

Chemicals and reagents

HPLC grade methanol, acetonitrile, and potassium dihydrogen orthophosphate, sodium hydroxide (A.R. grade) were obtained from Qualigen (Mumbai, India). Pure sample of drug and the commercial formulation (Tenvir-300 mg tablets) were obtained from Cipla Laboratories Ltd., Goa, India and Viread–300 mg tablets from Matrix Laboratories Ltd., Nasik, India, respectively. The molecular weight of tenofovir disoproxil is 519.44 (g/mol). High quality water, applied throughout the study, was obtained using a milli-Q® UF-PLUS apparatus (milli pore). All the excipients comply with the Pharmaceutical European requirements. The HDPE bottle and the polypropylene cap meet the general Pharmaceutical European requirements for plastic primary packaging material. Confirmation has been given that the silica gel desiccant canisters/sachets and the polyester fiber packing material are suitable for contact with food.

Instrumentation and analytical conditions

The HPLC system (Merck, Darmstadt, Germany) consisted of a pump (type l-6200 Intelligent pump) equipped with a Rheodyne Model 7161 injection valve with 20 µL loop (Rheodyne Inc., Cotati, CA, USA), a UV-detector (type L-4000) set at 260 nm, an integrator (type D-2500). The analytical column, Thermo Hypersil HyPURITY C18 reversed-phase column (150 mm × 2.1 mm i.d., 5 µm particle size) (Agilent Technologies, Massy, France) was operated at ambient temperature (20 ± 1^{o}C). Isocratic elution with acetonitrile and phosphate buffer adjusted to pH 6.0 (portable pH/mV meter PHM201 connected to

pHC3005-8 combined pH electrode) (Radiometer Analytical, Lyon, France) by 10% sodium hydroxide (50:50, v/v) was used at a flow rate of 1.0 mL/min. The mobile phase was prepared freshly, filtered through a 0.20 µm polyamide-membrane filter (Sartorius, Goettingen, Germany) using a glass vaccum filteration apparatus HetoMaster Jet type SUE 300Q (Heto Lab Equipment, Allerod, Denmark) and degassed by sonicating for 5 min before use (Ultrasonic's type TS540, Fisher Bioblock Scientific, kirch, France). The UV spectrum of tenofovir disoproxil for selecting the working wavelength of detection was taken using a Secomam S1000 PC UV-Vis spectrophotometer (Secomam, Domont, France).

Stock and working standard solutions

Stock standard solutions of 1 mg/mL of TD were prepared freshly by accurately weighing approximately 100 mg of TD in 100 mL volumetric flask. It was dissolved in 10 mL of methanol and and made up to volume with buffer pH 6.0 and sonicated for 10 min. These solutions were further diluted with buffer to obtain five working standards in the concentration range of 10, 20, 30, 40, 50 µg/mL of TD covering 60-140% of the intended test concentration of 30 µg/mL for the pharmaceutical formulation. All the solutions were prepared in triplicates. Before being subjected to analysis, all the working standard solutions were filtered through a 25 mm nylon membrane syringe filter (pore size 0.45 µm).

Before injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. 20 µL of each dilution was injected each time into the column at a flow rate of 1.0 mL/min. The calibration curve was plotted with the five concentrations of the10-50 µg/mL working standard solutions. So chromatography was repeated thrice for each dilution. Calibration solutions were prepared daily and analysed immediately after preparation and the corresponding chromatograms were obtained.

Assay sample preparation

The contents of twenty commercial tablets (labelled concentration 300 mg TD) were each weighed and their mean mass was determined. After homogenizing the powder by grinding in a glass mortar, an accurately weighed portion of the pooled sample equivalent to 100 mg was quantitatively transferred into a 100 mL volumetric flask containing 10 mL of methanol. The solution was sonicated for 15 min, to ensure the complete solubility of the drug. The mixture was then made upto 100 mL with buffer of pH 6.0. Appropriate aliquot was pippetted out from the stock solution and was further diluted with the mobile phase to obtain a concentration of 30 µg/mL. An aliquot of this solution was filtered through a 25 mm nylon membrane syringe filter (pore size 0.45 µm) prior to the injection into the HPLC system. Peak area ratios of TD were then measured for the determinations.

Validation procedure

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines[9]. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy, specificity, short-term stability and system suitability.

Standard plots were constructed with five concentrations in the range of 10-50 µg/mL prepared in triplicates to test linearity. The ratio of peak area signal of TD was plotted against the corresponding concentration to obtain the calibration graph. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method.

The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from six replicate injections of freshly prepared TD solution in the same equipment at a concentration of 100% (30 µg/mL) of the intended test concentration additionally on 2 consecutive days to determine intermediate precision. Peak area ratios of TD were determined and precision was reported as % R.S.D.

Method accuracy was tested (% recovery and % R.S.D. of individual measurements) by analyzing samples of TD at three different levels (60, 100 and 140%) in pure solutions using three preparations for each level. The results were expressed as the percentage of TD recovered in the samples.

Specificity was assessed by comparing the chromatograms obtained from sample of pharmaceutical preparation and standard solution with those obtained from excipients, which take part in the commercial tablets, and verifying the absence of interferences.

Sample solution short-term stability was tested at ambient temperature ($20 \pm 1^{o}C$) for 3 days. In order to confirm the stability of both; standard solution at 100% level and tablet sample solutions, both solutions protected from light were reinjected after 24 and 48 hr at ambient temperature and compared with freshly prepared solutions.

A system suitability test was performed by six replicate injections of the standard solution at a concentration of 30 µg/mL verifying TD resolution >2; % R. S. D. of peak area ratio of TD ±2%; % R. S. D. of each peak retention time ± 2%.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

Preliminary experiments have been performed on the basis of the RP-HPLC method described by Wiltshire et al.[10] TD is a lipophilic compound (Thermo Hypersil HyPURITY C18 and Shim-Pack ODS). It was tested to obtain optimized response of suitable retention time and good peak shapes for TD. The Thermo Hypersil HyPURITY C18 column was selected for all the analysis since it provided symmetrical peak shape and the highest intensity to TD. The separation of TD was affected by composition of the mobile phase on the retention time, which was thoroughly investigated. The concentration of the buffer (10-80 mM KH_2PO_4) as well as the amount of acetonitrile (50-70%, v/v) was optimized to give symmetric peak with short retention time. An improvement was observed in the retention time and in the stability of peak symmetry, when increasing the buffer concentration but it was important not to use as high concentration as 80 mM because of significant absorbance of KH_2PO_4 at the detection wavelength of 260 nm. KH_2PO_4 concentration of 50 mM was found to be suitable.

Changes in acetonitrile percentage from 50 to 65% resulted in decreasing the retention of TD but no significant change in peak symmetry was observed. On the other hand, increasing the amount of acetonitrile from 70% led to an increase of retention time; the optimum concentration was found to be 50%.

The effect of pH in the chromatographic elution of TD is related to the degree of ionization. TD is a basic and highly polar compound with low pK_a value 3.75. pK_a calculations on SYBYL and MOE software's show that TD (acyclic nucleoside phosphonate) is always under ionic form between pH 6 and 7. Variation of retention factor with pH is correlated with pK_a calculations[11]. Between pH 6 and 7, an increase of pH lead to increase of retention factor but decreasing resolution. This suggests that retention is mostly governed by reversed phase mechanism and selectivity increases with ionic interactions. At the optimum pH value (pH 6.0), it is positively charged, which leads to rapid elution with appropriate peak symmetry. Therefore, the pH value 6.0 was chosen for the optimum separation of the compound, as at this pH, the analyte peak were well defined with complete baseline resolution. The chromatograms recorded for bulk and formulation are shown in Fig. 2 and Fig. 3, respectively.

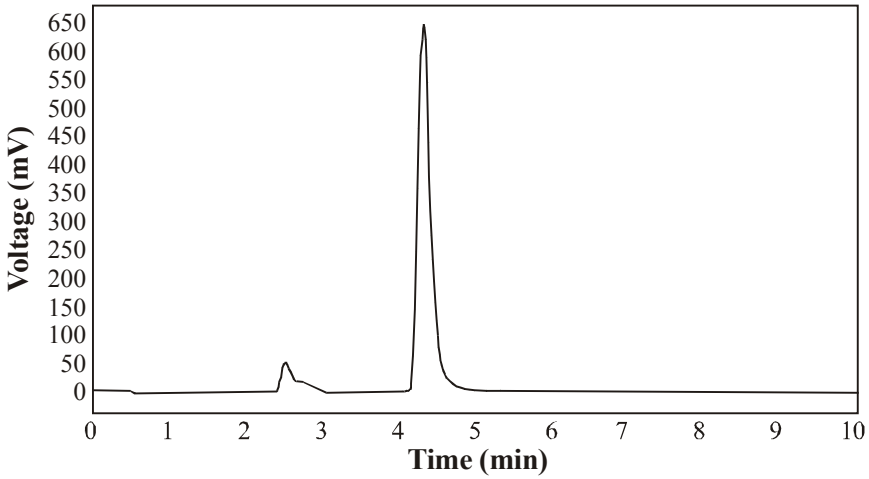

Fig. 2: Chromatogram of bulk drug TD (30 µg/mL). Retention time is 4.45 min

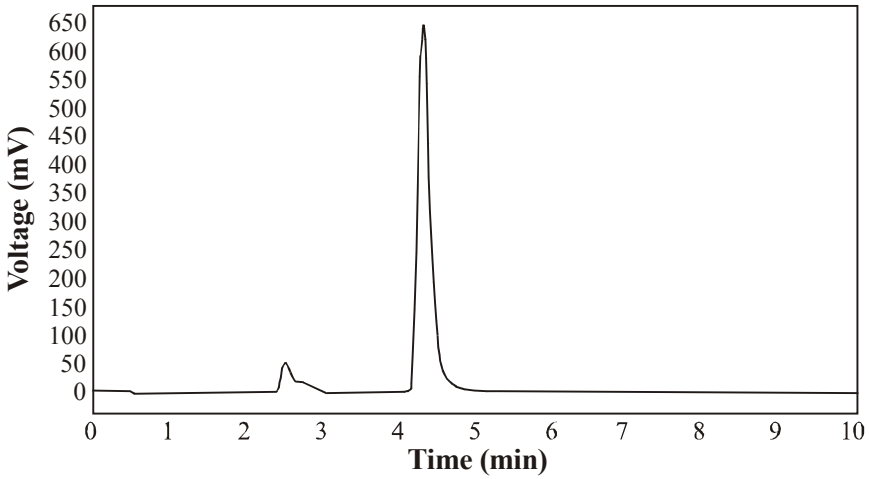

Fig. 3: Chromatogram of formulation TD (30 µg/mL). Retention time is 4.45 min

Validation of methods

Linearity

Five points calibration graphs were constructed covering a concentration range 10-50 µg/mL. Three independent determinations were performed at each concentration. Linear relationships of TD versus the corresponding drug concentration were observed, as shown by the results presented in Table 1. The standard deviations of the slope and intercept were low. The determination coefficient (r^2) exceeded 0.99. The calculated F-value equal to 2807

is highly significant. The limit of detection (LOD) is found to be 0.15 µg/mL and limit of quantification (LOQ) is 0.6 µg/mL, The calculation was based on the standard deviation of the response and the slope using the calibration curve.

Table 1: Statistical analysis of calibration curves in the HPLC determination of tenofovir disoproxil (n =15)

Validation parameters	**HPLC**
Concentration range (µg/mL)	10 – 50
Number of concentration levels	5
Regression equation	
Slope (b)	0.0397
Standard deviation on slope (S_b)	0.0007
Intercept (a)	0.0319
Standard deviation on intercept (S_a)	0.0393
Determination coefficient (r^2)	0.9954
F-value	2807
Limit of detection (LOD), µg/mL	0.15
Limit of quantification (LOQ), µg/mL	0.6

A Student's t-test was performed to determine whether the experimental intercept (a) of the regression equation was not significantly different from the theoretical zero value. It concerns the comparison of $t = a/S_a$, where a is the intercept of the regression equation and S_a is the standard deviation of a, with tabulated data of the t-distribution. As the calculated t-value (t = 0.81) does not exceed to (0.05, 13) = 2.16, the intercept of regression equation is not significantly different from 0.

Precision

The repeatability study (n = 6) carried out showed a R.S.D. of 0.858% for the peak area ratio of TD; thus, showing that the equipment used for the study worked correctly for the developed analytical method and being highly repetitive. For the intermediate precision, a study carried out by the same analyst working on 3 consecutive days (n = 3) indicated a R.

S. D. of 0.744%. Both values were far below 5%, the limit percentage set for the precision and indicated a good method from precision point of view.

Accuracy

The data for accuracy were expressed in terms of percentage recoveries of TD in the real samples. These results are summarized in Table 2. The mean recovery data of TD in real samples were within the range of 100.01 and 102.28%, mean% R. S. D. was 1.04%, satisfying the acceptance criteria for the study[12].

Table 2: Accuracy study for tenofovir disoproxil (n = 9)

Concentration levels (µg/mL)	Mean recovery (%)	R. S. D. (%)
10	100.01	0.81
30	101.74	1.67
50	102.28	0.65

Specificity

The HPLC chromatogram recorded for the separated drug excipients revealed no peak within a retention time range of 5 min. The results showed that the developed method was specific as none of the excepients interfered with the analytes of interest (Fig. 4).

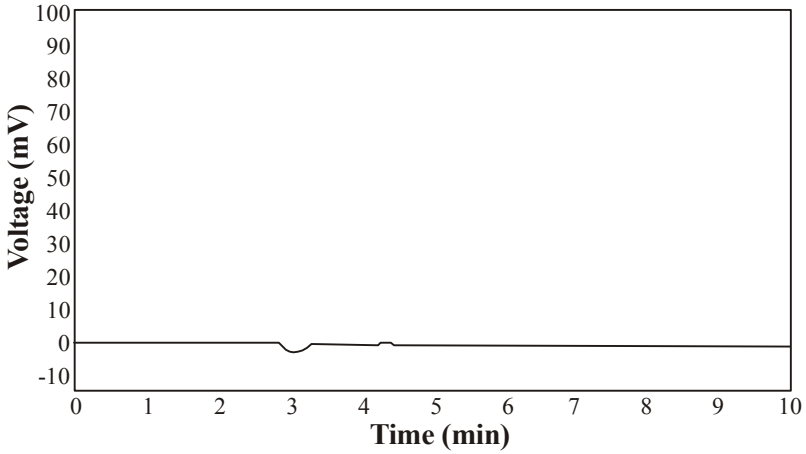

Fig. 4: Chromatogram of excipients of TD (30 µg/mL)

Stability

The stability of TD in standard and sample solutions was determined by storing the solutions at ambient temperature (20 ± 1°C) protected from light. The solutions were checked in triplicate after 3 successive days of storage and the data were compared with freshly prepared samples[13]. In each case, it could be noticed that solutions were stable for 72 hr, as during this time, the results did not decrease below 97%. This denotes that TD is stable in standard and sample solutions for atleast 3 days at ambient temperature and protected from light.

System suitability

The resolution factor in the developed method was above 1.5. The %R.S.D. of peak area ratios of TD and retention time were within 2% indicating the suitability of the system (Table 3). These results indicate the applicability of this method to routine with no problems and its suitability is proved. The statistical evaluation of the proposed method revealed good linearity, reproducibility and its validation for different parameters and led us to the conclude that it could be used for the rapid and reliable determination of TD in commercial pharmaceutical formulations.

Table 3: System suitability study

	Retention time (min)	Area ratio
	TD (30 µg/mL)	
Mean (n =10)	4.45	2.00
%R.S.D.	0.95	1.10

Assay of tablets

The validated method was applied for the assay of twenty commercial tablets containing 300 mg of TD: Viread, Tenvir. Each sample was analysed in triplicate after extracting the drug as mentioned in assay sample preparation of the experimental section and injections were carried out in triplicate. Fig. 2 shows RP-HPLC chromatogram of TD in pharmaceutical tablets. None of the tablet ingredient interfered with the analyte peak. The results presented in Table 4 are in good agreement with the labelled contents. Assay results, expressed as the percentage of the label claim, were found to be 100.5% for Viread, and 100.9% for Tenvir, showing that the content of TD in the tablets formulations conformed to

the content requirements (95-105%) of the label claim. Low values of standard deviation denoted very good reproducibility of the measurement.

Table 4: Results obtained for determination of tenofovir disoproxil in pharmaceutical formulation using the proposed HPLC method

Sample	TD (theoretical value) (mg/tablet)	TD (determined value)[a] (mg/tablet)	Recovery[a] (%)	R. S. D.[a] (%)
Viread	98.5	99.5	100.9	1.2
Tenvir	98.5	99.0	100.5	1.5

[a]Mean of nine determinations.

The above results demonstrated that the developed RP-HPLC method achieved rapid and accurate determination of TD and could be used for the determinations of TD in pharmaceutical formulations, as there was no interference from tablet excipients

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