Chromatographic Method for Quantification of Tenofovir in Pharmaceutical Formulations: Comparison with Spectrophotometric Method

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Authors’ contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: An efficient therapeutic drug or antiviral agent to treat Covid-19 is still not available. But, previously licensed pharmaceuticals to treat other virus infections are used on an off-label basis either alone or in combination. Tenofovir emerged as a promising treatment for Covid-19 in September 2020. It is an antiviral drug that is active against many viruses. This study explains the development and assessment of liquid chromatographic and UV spectrophotometric methods to quantify tenofovir in raw materials and tablets.

Methods: HPLC analyzes were performed using a C18 column and a mobile phase composed of 20 mM KH₂PO₄, with a flow rate of 0.8 mL/min and UV detection at 260 nm. For the spectrophotometric analyzes, ultra-pure water was used as a solvent. UV spectrum of standard and sample solution were recorded between 200 and 400 nm and Tenofovir was detected using a wavelength of 260 nm. Both methods have been validated according to the procedures described in ICH guidelines Q2(R1) for the validation of analytical methods.
Results: The results showed that both spectrophotometric and liquid chromatographic methods were linear, precise, accurate, rugged and robust with relative standard deviation R.S.D.% values less than 1%, and the recovery percentage was within standard limits (98-102%). Then a statistical comparison of these two analytical methods was performed, and the results of both methods showed no significant difference. It was found that both methods were not statistically significant with respect to each other in the 95% confidence interval (p<0.05). As a result, the proposed methods were found to be highly effective and could be used for routine analysis of tenofovir in pharmaceutical formulations.

Keywords: Tenofovir; UV; LC; method; development; validation; comparative.

1. INTRODUCTION

Globally, As of 24 September 2021, WHO has reported 4,724,876 deaths and 230,418,451 cases of Covid-19 [1]. There is currently no available therapeutic agent or antiviral drug for the treatment of Covid-19. With high mortality rates, Covid-19 disease is uncontrollable and has become a serious issue on a global scale. Urgently, effective and reliable treatment methods for Covid-19 patients should be researched and developed. The medical need to treat covid-19 patients meets existing anti-viral drugs [2].

Tenofovir has emerged as a promising drug for the treatment of Covid-19 infection in September 2020. Extension reaction and molecular docking studies show that tenofovir, remdesivir and other nucleoside reverse transcriptase inhibitors can be effective against Covid-19 by inhibiting RNA polymerase, as long as they achieve high intracellular concentration in target tissues [2-4]. Ferret infection models have shown that tenofovir reduces Covid-19 titers in nasal washes [5]. Observational studies have shown that the hospitalization rate for patients diagnosed with Covid-19, which is positive for the human immunodeficiency virus, taking tenofovir, is 50% lower [6-8]. Tenofovir is currently being evaluated in controlled randomized clinical trials for both treatment and prophylaxis of Covid-19 [9]. Given the promising clinical trial results for remdesivir, tenofovir is expected to be effective because the two drugs are from the same family of drugs [10-11]. The main difference between these drugs is that tenofovir can be administered orally, while remdeasivir requires daily intravenous infusion.

Tenofovir is a nucleotide analog. Chemical structure of tenofovir has been shown in Fig. 1. It is now marketed in an oral dosage form in Turkey (Ternavir®, Atabay Pharmaceuticals and Fine Chemicals Inc.).

<table>
<thead>
<tr>
<th>Fig. 1. Chemical structure of Tenofovir</th>
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</table>

According to a review of the literature, analytical methods based on LC chromatographic [12,13], HPTLC [14], and UV spectrometric [15] are available for the determination of tenofovir in different dosage forms. Some papers have described the analysis of tenofovir in plasma, based on HPLC [16,17], stability indicating [18]. However, there are no reported articles on a comparative study of liquid chromatographic and spectrophotometric methods for quantitation of tenofovir.

As a result, a simple and accurate spectrophotometric method and a precise and accurate chromatographic method were developed and validated. Both methods can be quite useful for routine analysis of drug formulations. The results of the analytical methods developed have been compared statistically by the analysis of variance.

2. MATERIAL AND METHODS

An HPLC system (Agilent 1260) was used during method development. This system consisted of Chemstation software, an UV-V detector, a quaternary pump, a degasser, an auto sampler, a thermostated column compartment. Shimadzu UV-1800 spectrophotometer with UV-Probe software and a doublebe amusing 1.0 cm quartz cells was also utilized in the present study.
Ultra-pure water used in experimental studies was produced in the Milli-Q water purification system (Millipore). Pure tenofovir and Ternavir (245 mg perfilm-coated tablet) were supplied from Atabay Pharmaceuticals and Fine Chemicals Inc. (Istanbul, Turkey). All solvents were HPLC grade from Merck (Germany). Potassium dihydrogen phosphate was from Merck (Germany).

Stock standard solution with a concentration of 500 µg mL\(^{-1}\) was prepared by dissolving the appropriate amount of pure tenofovir (Atabay Pharmaceuticals and Fine Chemicals Inc. Istanbul, Turkey) in ultra-pure water. Series dilutions of stock standard solution with ultra pure water were performed to obtain working standard solutions at concentrations of 10, 20, 30, 40, 50 and 60 µg mL\(^{-1}\).

The standard solutions (tenofovir concentration: 10-60 µg mL\(^{-1}\)) were filtered using 0.22 micron syringe filters. The solutions of standard and sample were injected into the chromatographic system with constant injection volume (20 µL). A calibration curve for chromatographic method was plotted between the peak area and the concentration. Slope and regression coefficient were determined from the calibration curve. The absorbance values of these standard solutions were measured in a spectrophotometer device at a wavelength of 260 nm. A calibration curve for spectrophotometric method was plotted between the absorbance and the concentration. It was found that the absorbance of standard solutions was proportional to the corresponding concentrations of tenofovir.

Five ternavir tablet were crushed into fine powder. Powder equivalent to 50 mg tenofovir was accurately weighed and dissolved in 50 mL of diluent in a 100 mL volumetric flask. The content of the flask was sonicated for ten min to dissolve the drugs completely followed by dilution up to the mark with the same solvent. Pipette 1.0 mL of the prepared solution into a 10 mL of volumetric flask and dilute up to the mark with diluent (concentration: 50 µg mL\(^{-1}\) tenofovir). 20 µL of the prepared tablet solution was injected (n=3) into the chromatographic system. Using the proposed method, the peak areas were measured for tenofovir and calculated their content in the tablet.

First, the spectrophotometer was zero-calibrated using ultra-pure water. Tenofovir standard solution (30 µg mL\(^{-1}\)) was then scanned in the range of 200 and 400 nm on the spectrophotometer to determine the wavelength at which the tenofovir absorbs maximally.

Chromatographic analyzes were performed on a liquid chromatography device (Agilent 1260) with a UV-Vis detector. 20 mM KH\(_2\)PO\(_4\) solution (pH 7.5) was used as mobile phase, the flow rate was determined as 0.8 mL min\(^{-1}\). Before use, the mobile phase was filtered using a 0.22 µm membrane filter and degassed in an ultrasonic bath. A C\(_{18}\) (4.6 mm x 250 mm, 5.0 µm particle size, Agilent) column was used and kept at 25 °C. Tenofovir was detected at 260 nm by UV detector. Under these conditions, the run time was 10 minutes. All absorbance measurements were performed at 260 nm using 1.0 cm quartz cells on a double beam spectrophotometer.

Both analytical methods have been validated according to the procedures described in ICH guidelines Q2(R1) for the validation of analytical methods [19,20]. Validation parameters (Linearity, accuracy precision, sensitivity, specificity, robustness, system suitability tests, stability studies) have been investigated.

Appropriate dilutions were performed from the tenofovir stock solution with ultra pure water to obtain concentrations in the linear range of 20-70 µg mL\(^{-1}\) for validation of the chromatographic method. Then, 20 µL of each diluted solution was injected into the HPLC system, peak area and retention time of tenofovir were recorded. The calibration curve for tenofovir was constructed by plotting the concentration of the standard solution against the peak area, and linearity was evaluated by the regression equation. The values of the correlation coefficient, intercept, and slope were recorded. Each experiment was performed in six replicates. Tenofovir standard solutions in the concentration range of 10-60 µg mL\(^{-1}\) were scanned in a wavelength range of 200-400 nm on a spectrophotometer device and \(\lambda_{\text{max}}\) was determined as 260 nm. Absorbance values of tenofovir standard solutions were determined at a wavelength of 260 nm in the spectrophotometer device. Six repetitions of the experiment were carried out. Standard addition method was followed to determine the method accuracy. A known amount of standards at three different levels (80%, 100% and 120% of the assay concentration) was added to the previously analyzed sample solution.

All the samples were analyzed by the developed methods. For each concentration, the relative
standard deviation (R.S.D.%) and recovery % were determined. For the UV spectrophotometric technique, absorbance was measured directly. The percentage of recovery was obtained by comparing the calculated and measured concentrations. The precision of the methods have been assessed by repeatability (intra-day) and intermediate precision (inter-day). Intra day precision studies were conducted by estimating the response sixtimes in the same day using three different concentrations (40, 50 and 60 µg mL\(^{-1}\)) of tenofovir and inter-day precision studies were performed by repeating the above-mentioned procedure on three consecutive days. Precision study findings were represented as a R.S.D.%

The detection limit (LOD) and quantification limit (LOQ) were used to evaluate the sensitivity of chromatographic and spectrophotometric methods (LOQ). The following equations were used to calculate the LOD and LOQ values from the calibration curve [19,20].

\[
\text{LOD} = 3.3\sigma/S \quad \text{and} \\
\text{LOQ} = 10\sigma/S, \quad \text{where}, \\
\sigma = \text{standard deviation of y intercept of calibration curve} \quad \text{S} = \text{slope of the calibration curve}
\]

For Specificity, the purity of the tenofovir peak was determined by comparing the individual chromatograms in the HPLC method in three regions: start, apex, and end of the peak.

The robustness of both analytical methods was assessed by examining the effect of small but deliberate changes in method parameters on the analysis results. To test the robustness of the chromatographic method, the same sample was analyzed under different conditions such as changes in the mobile phase flow rate (±0.1 mL min\(^{-1}\)) and detection wavelengths (±2 nm), and the effect on the system suitability parameters has been observed. To test the robustness of the spectrophotometric method, the same sample was analyzed under different conditions, such as changes in the solvents used and changes in the detection wavelengths and the effect on the system suitability parameters has been observed.

For system suitability tests; The standard solution of tenofovir (30 µg mL\(^{-1}\)) in concentration was injected six times. Tenofovir peak areas, theoretical plate numbers (N), queuing factors (T), capacity factors (k'), and asymmetric factor (As) were evaluated.

For stability studies; A tenofovir solution in ultrapure water was stored for up to two days under laboratory bench conditions and for five days under refrigeration (4±0.2 °C). Then, these solutions were analyzed with the developed methods.

Both analytical methods were found to be appropriate for quantification of tenofovir in pharmaceuticals after validation. When both analytical methods were used on commercial pharmaceuticals, the recovery percentages were compared statistically. The F-test and t-test were used for this purpose.

### 3. RESULTS AND DISCUSSION

In this study, a new chromatographic method and spectrophotometric method for the determination of tenofovir in pharmaceutical formulations has been defined.

Chromatographic conditions were carefully optimized. The composition and flow rate of the mobile phase were determined considering the peak shape (symmetry, tailing factor), analysis time, cost of solvents, and baseline drift. Many mobile phases were tried to achieve excellent separation. Finally, 20 mM KH\(_2\)PO\(_4\) at pH:7.50 was found to be the best mobile phase for an acceptable K value, short run time, and symmetric peak.

Fig. 2 shows overlap chromatograms of standard solutions, Fig. 3 shows linearity graph of standard solutions for chromatographic method. The retention time of tenofovir was 2.27 min. The developed method was validated in terms of linearity, accuracy, specificity, precision, sensitivity (LOD and LOQ), system suitability, and robustness by following ICH guidelines recommendations.

The peak purity index of tenofovir was determined as 1 and the method was observed to be specific. The peak area and the analyte concentration have an excellent correlation according to linear regression analysis. Over the concentration range of 20 to 70 µg mL\(^{-1}\), calibration curve for the standards was linear with a correlation coefficient (r\(^2\)) of 0.9999±0.00022, the corresponding linear regression equation being \(y = 1.9331x + 2.8419\). LOD and LOQ were 2.30 µg mL\(^{-1}\) and 6.90 µg mL\(^{-1}\) respectively, showing the method's sensitivity.
The high accuracy and precision of the method is evident from the results and statistical evaluations shown in Table 1. Experiments on recovery showed good accuracy with low relative standard deviations (R.S.D.%). The developed HPLC method was found to be precise because R.S.D.% values were <2 for repeatability and intermediate precision studies as recommended in the ICH guidelines. R.S.D.% of repeatability (intra-day) and intermediate precision (inter-day) ranged from 0.0940 to 0.1515 and 0.1840 to 0.2615 respectively. The regression and validation parameters of tenofovir using both methods were shown in Table 1.

During these experiments, deliberate modifications in experimental circumstances had no effect on peak symmetry, and there was no statistically significant difference in tenofovir retention time. The R.S.D. % were calculated for each method parameter and were found to be < 2. The percentage amount of tenofovir present in the assayed sample was found to be 100.055±0.0512. System suitability tests ensure the suitability of the suggested HPLC method for routine tenofovir analysis. The capacity factor (k’) of the tenofovir peak was determined as 11.84.

### Table 1. Validation and regression parameters for analytical methods (n=6)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spectrophotometric method</th>
<th>Chromatographic method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration range (μg mL⁻¹)</td>
<td>10-60</td>
<td>20-70</td>
</tr>
<tr>
<td>Limit of detection and quantification (μg mL⁻¹)</td>
<td>2.6/7.9</td>
<td>2.3/6.9</td>
</tr>
<tr>
<td>Slope</td>
<td>0.02900±0.0029</td>
<td>0.19331±0.0016</td>
</tr>
<tr>
<td>Standard Error (Slope)</td>
<td>0.0230</td>
<td>0.2700</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0191±0.0056</td>
<td>2.8419±0.0042</td>
</tr>
<tr>
<td>Standard Error (Intercept)</td>
<td>0.0094</td>
<td>0.5430</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>0.9996±0.0008</td>
<td>0.9999±0.0005</td>
</tr>
<tr>
<td>Standard deviation (Residuals)</td>
<td>1.38</td>
<td>0.51</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>100.25±0.2080</td>
<td>100.20±0.1060</td>
</tr>
<tr>
<td>Intra-day precision (R.S.D. %)</td>
<td>0.2120-0.3430</td>
<td>0.0940-0.1515</td>
</tr>
<tr>
<td>Inter-day precision (R.S.D. %)</td>
<td>0.2520-0.4530</td>
<td>0.1840-0.2615</td>
</tr>
</tbody>
</table>

**Fig. 2. Overlap chromatograms of standard solutions for LC method**
It was observed that the areas of the peaks obtained from the chromatograms increased proportionally as the concentrations of the standard solutions increased.

The correlation coefficient of the line obtained from the concentration vs. peak area plot was determined as 0.9999. This indicates that both peaks are well resolved according to the void volume. The tenofovir peak has a tailing factor of 1.355, indicating strong peak symmetry. The theoretical plate number (N) for the tenofovir peak was determined to be 6542, indicating high column efficiency. The system suitability tests (Table 2) reveal that the developed method meets these requirements with in acceptable ranges. Solution stability studies have shown that standard solutions are stable at room conditions.

Table 2. System suitability parameters of chromatographic method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>2.27</td>
</tr>
<tr>
<td>Peak purity index</td>
<td>0.9999</td>
</tr>
<tr>
<td>Asymetry</td>
<td>1.351</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.387</td>
</tr>
<tr>
<td>Capacity factor (k')</td>
<td>11.848</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>6542</td>
</tr>
</tbody>
</table>

Standard solutions prepared by making appropriate dilutions from the stock solution were
scanned in the wavelength range of 200-400 nm. Tenofovir exhibited maximum absorption at 260 nm. Fig. 4 shows overlap spectra of standard solutions, Fig. 5 shows linearity graphs of standard solutions for spectrophotometric method. The spectrophotometric method was validated according to ICH guidelines. Tenofovir in ultra-pure water demonstrated linear relationship in the concentration range of 10 to 60 μg mL⁻¹. Correlation coefficient ($r^2$) was 0.9997. The regression equation was found to be $y = 0.029x - 0.0183$. Tenofovir recoveries were determined using the standard addition technique for the three concentrations and ranged from 99.90% to 100.18% percent with admissible RSD values. LOD and LOQ were determined as 2.60 μg mL⁻¹ and 7.90 μg mL⁻¹ respectively (Table 1). Assay results varied from 99.92% to 100.14% with satisfactory RSD values.

The developed spectrophotometric method provided for the rapid quantification of tenofovir in pharmaceutical formulations. Because of its high solubility and stability, ultra-pure water was chosen as the solvent. Also, the spectral properties were good for this solvent. Low values of R.S.D. % indicate the precision of the method. The method was shown to be quite accurate, with mean recoveries close to 100%. Furthermore, as demonstrated by the LOD and LOQ values, the method is very sensitive.

Both analytical methods were applied to commercial pharmaceutical preparations. Analysis results for the Ternavin tablet containing tenofovir are presented in Table 3. It was observed that the results of the analysis were very close to the amounts stated on the labels of the tablets.

Both analytical methods were statistically compared using the F-test and the t-test. Statistical testing indicated that the experimental results obtained from the analyses performed by both analytical methods were not significantly different. At a 95% confidence interval, the calculated F-value and t-value were lower than the tabular values of both analytical methods. Both analytical methods proposed in this study appeared to be applicable to the appropriate Tenofovir quantification in pharmaceutical formulations. Data on the statistical comparison results of both analytical methods are presented in Table 4.

To assess the quantity of tenofovir in pharmaceutical formulations, two distinct analytical methods were established in this study: chromatographic and spectrophotometric methods. The spectrophotometric and chromatographic conditions of these developed methods have been optimized and validated.

![Figure 5. Linearity graphs of standard solutions for spectrophotometric method](image)

**Table 3. Application results of analytical method**

<table>
<thead>
<tr>
<th>Commercial Formulation</th>
<th>Label claim (mg/tablet)</th>
<th>Spectrophotometric method</th>
<th>Chromatographic method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found Tenofovir (mg/tablet)</td>
<td>Assay % ± S.D.</td>
<td>Found Tenofovir (mg/tablet)</td>
</tr>
<tr>
<td>Ternavin</td>
<td>245</td>
<td>245.97</td>
<td>100.40 ± 0.45</td>
</tr>
</tbody>
</table>
Table 4. Statistical comparison results of analytical methods (α:0.05, C.I: 95%, n:6)

<table>
<thead>
<tr>
<th>Statistical values</th>
<th>Chromatographic Method</th>
<th>Spectrophotometric Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay average</td>
<td>99.61</td>
<td>99.53</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.51</td>
<td>1.38</td>
</tr>
<tr>
<td>R.S.D.%</td>
<td>0.51</td>
<td>1.39</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.31</td>
<td>0.82</td>
</tr>
<tr>
<td>F-test</td>
<td>0.17/0.23</td>
<td></td>
</tr>
<tr>
<td>Fcalculation/Ftable</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.17</td>
<td>0.04</td>
</tr>
<tr>
<td>t-test</td>
<td>1.92/2.71</td>
<td></td>
</tr>
<tr>
<td>tcalculation/ttable</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A variety of analytical methods for quantifying tenofovir in pharmaceutical formulations have been reported. Some of these techniques are complicated. These procedures need the use of costly apparatus, huge quantities of organic solvents, and specialized reagents. The analysis takes a long time. Furthermore, the spectrophotometric and spectrofluorimetric techniques described in the literature need complicated and time-consuming sample preparation processes. There has yet to be a research in which two distinct analytic methods were created and statistically compared in any of these investigations.

4. CONCLUSION

Both spectrophotometric and chromatographic methods developed in this study did not use chemicals harmful for environment and human health. Both methods are economical. Spectrophotometric techniques do not often need complicated operations or processes. It is more cost-effective and takes less time. These are advantages of spectrophotometric method over chromatographic method. Compared statistically, the chromatographic method is more accurate and precise than the spectrophotometric method. The findings suggest that both methods are appropriate for determining the amount of tenofovir in pharmaceutical formulations. Excipients in pharmaceutical preparations did not adversely affect the results of the analysis. Because both proposed methods are specific, simple, fast, precise and accurate, they can be successfully applied for routine quality control analysis in pharmaceutica dosage forms of tenofovir.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


