Selective RP-HPLC DAD Method for Determination of Tenofovir Fumarate and Emtricitabine in Bulk Powder and in Tablets

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Summary. A simple and selective liquid chromatographic method was developed for the simultaneous determination of tenofovir disoproxil fumarate (TEN) and emtricitabine (EMT) in combined tablets. The method is based on separation of TEN and EMT on a Zorbax SB-C8 column, 5 μm, 4.6 × 250 mm, with a mobile phase consisting of 50 mM disodium hydrogen phosphate–acetonitrile (50:50, v/v). The mobile phase contains 0.1% triethylamine (TEA) and was adjusted to pH 6.0. Quantification of the analytes is achieved with diode array–ultraviolet detector (DAD–UV) at 260 and 280 nm for TEN and EMT, respectively, based on peak area. Different variables affecting the method were carefully investigated and optimized. Reliability and analytical performance of the proposed method, including linearity, range, precision, accuracy, and detection and quantitation limits, were statistically validated. The high-performance liquid chromatography (HPLC) method was successfully applied for determination of each drug in their binary tablets.

Key Words: HPLC, DAD, tenofovir, emtricitabine, tablets

Introduction

Antiviral drugs development has become a very active area in the last decade, especially with the challenges of AIDS, hepatitis, avian, and swine flu epidemics. The antiviral drugs are used in the treatment of viral infections. They may also be used to provide protection, usually for a brief period only, against infection. There is little evidence that these compounds affect latent or nonreplicating virus. Nonspecific symptomatic and supportive treatment is also important in the management of viral infections.

Tenofovir disoproxil fumarate (TEN) is 9-[(R)-2 [[bis[[[(isopropoxy-carbonyl) oxy]methoxy] phosphinyl] methoxy] propyl] adenine fumarate [1] (Fig. 1). It belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, an enzyme crucial to viral production in human immunodeficiency virus (HIV)-infected people [2, 3].
Emtricitabine, 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one [1] (Fig. 1), works by inhibiting reverse transcriptase enzyme that copies HIV RNA into new viral DNA. It can help to lower the level of HIV in the patient’s body and can indirectly increase the number of immune system cells. Emtricitabine (EMT) is indicated in combination with other antiretroviral agents for the treatment of HIV and hepatitis B virus (HBV) infection in adults [2, 3]. Being relatively recent drugs, TEN and EMT are not official in British Pharmacopoeia 2010 (BP 2010) or United States Pharmacopoeia (USP 2011).

![Fig. 1. Structures of tenofovir disproxyl fumarate (TEN) and emtricitabine (EMT)](image)

TEN is formulated in binary mixture with the reverse transcriptase inhibitor EMT to prevent HIV from altering the genetic material of healthy T-cells. Combining the two drugs in one tablet helps reduction of the pill burden and increases the compliance with antiretroviral therapy.

Simple spectrophotometric techniques have been published involving determination of TEN by direct methods [4, 5] and derivative calculation [6–8]. Other spectrophotometric methods published include colorimetric assays using various derivatizing reagents [9–12].

High-performance liquid chromatography (HPLC) finds great application in the therapeutic drug monitoring of TEN in different biological samples [13–19]. Almost all HPLC assays depend on mass spectrometric detection of TEN. Only one method made use of ultraviolet (UV) for detection of TEN at 259 nm [15]. Other HPLC methods are applied for assay of TEN in bulk powder and in pharmaceutical preparations [20–22].

The literature reveals that HPLC is the most common technique for the analysis of EMT. Several HPLC methods aiming at the determination of
EMT in plasma were found in the literature depending on either UV detection [23, 24] or fluorometric detection [25]. The quality control of pharmaceutical products could be monitored using a published HPLC methodology [26]. Few LC methods were developed for determination of EMT and its related compounds [27–29]. The analytical behavior of EMT in gas chromatography and polarography was also studied [30]. Several HPLC methods were found in the literature for the analysis of TEN–EMT mixture [31–34]. Detection of both drugs in these methods was achieved either depending on UV-detection [31, 33, 34] or mass spectrometry [32, 35, 36]. The reported methods were applied for the simultaneous determination of TEN and EMT in plasma [31, 32, 35, 36] or in pharmaceuticals [33, 34]. Two spectrophotometric methods were published for the assay of the binary mixture in tablets [37, 38]. Only one HPTLC method was found for the determination of the mixture in tablets using chloroform and methanol (9:1) as a mobile phase and UV detection at 265 nm [39].

The literature reveals few methods for the simultaneous quantitation of TEN–EMT mixture with other antiviral drugs as lamivudine and/or zidovudine [40, 41] and lopinavir [42] using HPLC technique.

**Experimental**

**Instrumentation**

The HPLC–diode array detector (DAD) system consisted of Agilent (Santa Clara, CA) 1200 series (quaternary pump, vacuum degasser, and diode array and multiple wavelength detector G1315 and G 1365 C/D) connected to a computer loaded with Agilent ChemStation software. A Rheodyne manual injector (USA) with 20-μL loop was used. The column used was Zorbax SB-C8 column, 5 μm, 4.6 × 250 mm (Agilent).

**Materials and Reagents**

Authentic samples of TEN and EMT were kindly provided by Gilead Pharmaceuticals, USA. Analytical grade of Na₂HPO₄ and TEA were used. HPLC grade acetonitrile (LAB-SCAN analytical sciences, Poland) was used. Truvada® tablets are labeled to contain 300 mg TEN and 200 mg EMT (Gilead Sciences Inc., Canada).

Stock solutions of either TEN or EMT 1000 μg mL⁻¹ were prepared in high purity distilled water and stored refrigerated at 4 °C.
General Procedure and Construction of Calibration Curves

The mobile phase was prepared by mixing acetonitrile:50 mM Na₂HPO₄-TEA (50:50:0.1, v/v/v) and then adjusting the pH value at 6.0. The mobile phase was filtered and degassed by passing through a 0.45-μm pore size membrane filter prior to use. The flow rate was 0.4 mL min⁻¹, the injection volume was 20 μL, the eluent was monitored using the diode array detector (DAD) from 190 nm to 400 nm, and chromatograms were recorded at 260 nm for TEN, 260 nm and 280 nm for EMT. All determinations were performed at ambient temperature.

Accurate volumes of TEN and EMT stock solutions were diluted with the mobile phase in 10-mL volumetric flasks to reach concentration ranges of 0.3–100 μg mL⁻¹ for TEN and 0.8–80 μg mL⁻¹ for EMT. Triplicate injections were made for each concentration and chromatographed under the justified LC conditions. The peak areas for the investigated drugs were plotted against the corresponding concentrations to construct the calibration graphs and regression equations for both drugs were computed.

Validation of the Developed HPLC Method

System Suitability

The system suitability parameters were evaluated by using a solution of TEN and EMT (10 and 20 μg mL⁻¹, respectively, in the mobile phase). Six replicates were injected to the HPLC system. The linearity of the method was evaluated by constructing calibration curves as described above. Calibration curves were constructed by plotting the measured peak area over concentration of standard samples, and statistical analysis was performed.

Accuracy and Precision

The within-day and between-day accuracy and precision were established by using three replicates of standard solutions of each drug at three different concentrations (10, 50, and 100 μg mL⁻¹ for TEN; and 5, 20, and 80 μg mL⁻¹ for EMT) during a single day and three separate days.
Selectivity

Several laboratory-prepared mixtures of the TEN and EMT at different concentrations within the linearity ranges mentioned in Table I were prepared and chromatographed as under the general procedure.

Table I. Validation data for the determination of TEN and EMT using the proposed HPLC method

<table>
<thead>
<tr>
<th>Item</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEN (at 260 nm)</td>
</tr>
<tr>
<td>Concentration range (μg mL⁻¹)</td>
<td>0.3–100</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.99996</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>38.532</td>
</tr>
<tr>
<td>%y-intercept</td>
<td>0.831</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>155.449</td>
</tr>
<tr>
<td>RSD % of slope</td>
<td>0.255</td>
</tr>
<tr>
<td>$S_a$</td>
<td>15.161</td>
</tr>
<tr>
<td>$S_b$</td>
<td>0.397</td>
</tr>
<tr>
<td>$S_{y/x}$</td>
<td>46.166</td>
</tr>
<tr>
<td>LOD (μg mL⁻¹)</td>
<td>0.034</td>
</tr>
<tr>
<td>LOQ (μg mL⁻¹)</td>
<td>0.114</td>
</tr>
</tbody>
</table>

Robustness

By introducing small changes in the chromatographic parameters, the effects of the results were examined. The flow rate of the mobile phase was 1.0 mL min⁻¹. To study the effect of flow rate, the flow was changed by 0.1 units, i.e., from 0.9 to 1.1 mL min⁻¹, and the effect of acetonitrile concentration was studied ±2% from the nominal concentration and the source of acetonitrile was varied. The pHs values of the mobile phase studied were 5.9 and 6.1 instead of 6.0.

Stability

The stability of TEN and EMT standard solutions (10 μg mL⁻¹ in the mobile phase) was checked after 6 h at room temperature, and after storing at room temperature for 1 week. The concentration of each drug sample was determined and compared with freshly prepared samples.
Applications

Preparation and Analysis of TEN–EMT Binary Tablets Sample Solution

Truvada® tablets were weighed, massed, and finely powdered. An accurately weighed quantity of the powder equivalent to the average weight per tablet was transferred into a beaker containing 50 mL high purity distilled water. After sonication for 15 min, the content was filtered quantitatively into a 100-mL volumetric flask and diluted to volume using distilled water.

Aliquots of the TEN–EMT binary tablets sample solution were diluted with the mobile phase to obtain final concentrations within the specified ranges and then treated as under “General Procedure.”

Results and Discussion

Optimization of Chromatographic Conditions

A simple, rapid, and selective HPLC–DAD method has been developed for the separation and simultaneous determination of TEN and EMT in their binary tablets. Both drugs were baseline separated on a RP-C8 column with a mobile phase consisting of 50 mM Na₂HPO₄:acetonitrile (50:50, v/v, 0.1% TEA) adjusted to pH 6.0, in an isocratic mode and within a run time less than 8 min (Fig. 2). The most important aspect in LC method development is the achievement of sufficient resolution with acceptable peak symmetry. To optimize the assay parameters, several reversed-phase columns, Zorbax SB-C18 (4.6 × 250 mm), Zorbax Eclipse XDB-C18 (4.6 × 150 mm), and Zorbax SB-C8 (4.6 × 250 mm), were tested. The last column was found optimum as it gave the best resolution of the two drugs. Accordingly, it was chosen as the working column for this study.

Several mobile phases were evaluated using various proportions of different aqueous phases, and organic modifiers were adjusted at various pH values. An optimum stationary phase-mobile phase matching was established using the mobile phase combination of 50 mM Na₂HPO₄ solution and acetonitrile. Methanol and tetrahydrofuran were tried as organic modifiers, and Na₂HPO₄ solution was substituted by other aqueous phases, such as phosphoric acid or potassium dihydrogen phosphate. In these trials, TEN and EMT showed either broad asymmetric or overlapped peaks.

The effect of mobile phase pH was investigated within the range 4–6 at 0.5 pH unit interval, and it was found that, below pH 6.0, both drugs eluted as either broad asymmetric or forked peaks. This was explained by the relatively low pKₐ values of TEN and EMT (3.75 and 2.65, respectively) [43].
Above pH 6.0, TEN and EMT are almost completely ionized; hence, it is eluted symmetrically. TEA was added at a concentration of 0.1% v/v in order to improve the symmetry and sharpness of the eluted peaks especially that of EMT, thus, improving the performance and system suitability parameters of the chromatographic separation.

Fig. 2. Typical HPLC chromatogram of a 20-μL injection of 30 μg mL⁻¹ TEN and 20 μg mL⁻¹ EMT at 260 nm (A) and 280 nm (B)
The effect of flow rate was studied, and 0.4 mL min\(^{-1}\) was found optimum regarding run time, peak asymmetry, and column pressure. Quantification was performed using DAD based on peak area measurements. DAD enhances the power of HPLC and is an elegant option for assessing method specificity by comparison of recorded spectra during peak elution. In addition, the multiple-wavelength detector offers the advantage of measuring each analyte at its specific wavelength, thus, improving sensitivity. This is important in case of analytes with different absorption characteristics. TEN exhibits maximum absorbance at 260 nm, while EMT shows two maxima at 236 nm and 280 nm. EMT was quantified using the two detection wavelengths (260 nm and 280 nm). Yet, results at 280 nm were favored due to higher sensitivity in detecting the investigated compound.

**Method Validation**

The above described chromatographic conditions showed good separation between TEN and EMT within acceptable run time. *Figure 2* shows a typical chromatogram for the separation of TEN and EMT eluted at retention times of 4.98 ± 0.04 and 5.99 ± 0.09 min, respectively. Retention factors \((k')\) are 10.07 and 12.31 for TEN and EMT, respectively. A value of 1.5 for resolution implies a complete separation between two consecutive peaks [44]; resolution \((R_s)\) for the mixture under analysis is 3.96. Finally, column performance (apparent efficiency) can be expressed by the number of theoretical plates \((N)\), which equals 6784 and 7644 for TEN and EMT, respectively.

Validation of the method was achieved according to the International Conference on Harmonization (ICH) [45] validation items; the linearity of the proposed HPLC–DAD procedure was evaluated by analyzing a series of different concentrations for each drug substance. The responses measured at the specified wavelengths were found to be proportional to the drug concentration. *Table I* presents the validation data and statistical parameters for the proposed methods including linear regression equations, concentration ranges, correlation coefficients, standard deviation of the intercept \((S_a)\), the slope \((S_b)\), and standard deviation of residuals \((S_{y/x})\). Regression analysis shows good linearity as shown from the correlation coefficient values \((r > 0.99995)\), percentage relative standard deviation (RSD %) of the slope values which were found less than 0.5%, and the %\(y\)-intercept values which were found less than 1%. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated in accordance to the ICH guidelines based on signal-to-noise ratio values [45]. LOD and LOQ values were 0.034 μg mL\(^{-1}\) and 0.114 μg mL\(^{-1}\) for TEN, and 0.091 μg mL\(^{-1}\) and 0.303 μg mL\(^{-1}\) for EMT at 280 nm, respectively (*Table I*), which confirm the sensitivity of the pro-
posed HPLC method. The within-day precision and accuracy for the described methods were examined at three concentration levels using three replicate determinations for each concentration through the same day. Similarly, the between-day precision and accuracy were tested by analyzing the same three concentrations for each drug substance using three replicate determinations repeated on 3 days. Recoveries were calculated using the corresponding regression equations, and the results were satisfactory (Table II). The RSD % and relative error (Er) did not exceed 1%, proving the high repeatability and accuracy of the developed methods for the estimation of both analytes in their bulk form. The robustness of the developed method in case of HPLC was evaluated through making slight variations in the experimental conditions such as flow rate (±0.1 mL min$^{-1}$), RSD % = 0.183 and 0.553 for TEN and EMT, respectively; source of acetonitrile (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland or LabScan Analytical Sciences, Dublin, Ireland), RSD % = 0.059 and 0.154 for TEN and EMT, respectively; and pH of the mobile phase (±0.1 pH unit), RSD % = 0.625 and 0.857 for TEN and EMT, respectively. These variations did not have any significant effect on the measured peak areas for both drugs or on the chromatographic resolution. RSD % for the measured peak areas after these variations did not exceed 1%. Method selectivity was examined by preparing several laboratory-prepared mixtures of the two compounds at different concentrations within the linearity ranges mentioned in Table I. These mixtures had ratios both above and below the normal ratio expected in the tablets. The laboratory-prepared mixtures were analyzed according to the previously described conditions for each method. The recovery values, the percentage relative standard deviation (RSD %) and the percentage relative error values (Er %) stated in Table III, were satisfactory, thus, confirming the selectivity of the method and demonstrating its capability to resolve and quantify both analytes in different ratios. Hence, the results tabulated were satisfactory, indicating the selectivity of the proposed method. Furthermore, selectivity was also demonstrated by the separation of both analytes from formulation additives. The use of DAD enabled confirming the peak purity of the eluted compounds and the method selectivity; purity spectra are presented in Fig. 3. Peak purity was assessed by recording the absorption spectra of the eluted compounds from laboratory-prepared mixtures and binary tablet sample solution, and comparing them with the spectra of reference samples of TEN and EMT. It was clear that both analytes were eluted individually. Hence, the method is found capable of resolving TEN and EMT in their mixtures. The stability of TEN and EMT standard and sample working solutions in the mobile phase was verified during handling by keeping them at room temperature for 6 h. No chromatographic changes involving the peak areas or the resolution of both drugs at their specific retention times were
noticed. The stock solutions were also stable when kept refrigerated at 4 °C for at least 1 week. Comparing the developed method with two reports published concerning the LC determination of both drugs in tablets [33, 34]

Table II. Accuracy and precision of the proposed HPLC method for analysis of TEN and EMT

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal value (μg mL⁻¹)</th>
<th>Within-day (n = 9)</th>
<th>Between-day (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found ± SD (μg mL⁻¹)</td>
<td>RSD (%)</td>
<td>Er (%)</td>
</tr>
<tr>
<td>TEN</td>
<td>10.0</td>
<td>10.048 ± 0.027</td>
<td>0.266</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>49.842 ± 0.343</td>
<td>0.689</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>100.252 ± 0.102</td>
<td>0.102</td>
</tr>
<tr>
<td>EMT</td>
<td>5.0</td>
<td>4.973 ± 0.019</td>
<td>0.379</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.066 ± 0.099</td>
<td>0.494</td>
</tr>
<tr>
<td></td>
<td>80.0</td>
<td>80.475 ± 0.150</td>
<td>0.186</td>
</tr>
</tbody>
</table>

Table III. Determination of TEN and EMT in laboratory-prepared mixtures using the proposed HPLC method

<table>
<thead>
<tr>
<th>Nominal value (μg mL⁻¹)</th>
<th>Found ± SDa (μg mL⁻¹)</th>
<th>RSD %b</th>
<th>Er %c</th>
<th>TEN</th>
<th>EMT</th>
<th>TEN</th>
<th>EMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEN</td>
<td>10.0</td>
<td>10.017 ± 0.096</td>
<td>0.958</td>
<td>0.166</td>
<td>1.378</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMT</td>
<td>20.0</td>
<td>20.276 ± 0.072</td>
<td>0.919</td>
<td>0.591</td>
<td>−0.783</td>
<td>1.507</td>
<td></td>
</tr>
<tr>
<td>TEN</td>
<td>10.0</td>
<td>9.922 ± 0.091</td>
<td>10.083 ± 0.165</td>
<td>0.954</td>
<td>0.926</td>
<td>0.992</td>
<td>0.819</td>
</tr>
<tr>
<td>EMT</td>
<td>20.0</td>
<td>10.082 ± 0.093</td>
<td>20.326 ± 0.111</td>
<td>10.641</td>
<td>1.629</td>
<td>0.827</td>
<td></td>
</tr>
<tr>
<td>TEN</td>
<td>30.0</td>
<td>30.286 ± 0.220</td>
<td>30.298 ± 0.289</td>
<td>0.725</td>
<td>1.831</td>
<td>0.953</td>
<td>1.501</td>
</tr>
<tr>
<td>EMT</td>
<td>20.0</td>
<td>30.300 ± 0.372</td>
<td>10.036 ± 0.111</td>
<td>0.926</td>
<td>0.954</td>
<td>1.378</td>
<td></td>
</tr>
</tbody>
</table>

aMean ± standard deviation for five determinations.
b% Relative standard deviation.
c% Relative error.

Table IV. Application of the proposed methods to analysis of TEN and EMT in Truvada® tablets

<table>
<thead>
<tr>
<th>Drug</th>
<th>Method</th>
<th>HPLC method</th>
<th>Comparison reference methodb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEN</td>
<td>100.700 ± 0.69</td>
<td>100.46 ± 0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$t = 0.442, F = 2.10$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMT</td>
<td>99.98 ± 0.49</td>
<td>99.76 ± 0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$t = 0.669, F = 1.19$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aMean % recovery ± SD for five determinations.
bReference spectrophotometric method [46].
cTheoretical values for $t$ and $F$ at $P = 0.05$ are 2.31 and 6.39, respectively.
revealed that our proposed method utilised less organic solvent in the mobile phase used, and still, their $t_R$ is quite acceptable (less than 7 min). The proposed method made use of DAD as a tool for detection and assessing peak purity of both drugs, while the mentioned reports used conventional UV detection. Moreover, the proposed method is more sensitive than both reports. LOD and LOQ are more sensitive than the reported method [33] for TEN while they are comparable to the published report [34]; however, con-
cerning EMT, the LOD and LOQ of the published reports are more sensitive.

**Assay of Tablets**

The applicability of the validated HPLC method to the assay of pharmaceutical preparations was examined by analyzing Truvada® tablets. Results obtained using the proposed method (*Table IV*) were compared to those obtained using a literature reported spectrophotometric method [46]. The calculated Student’s *t*-test and the variance ratio *F*-test did not exceed the theoretical ones which indicated that there was no significant difference between the investigated methods. Moreover, no interfering peaks were observed in the liquid chromatogram of the tablet sample solution (*Fig. 4*) which confirmed the selectivity of the method. Moreover, there was no interference from the excipients present in the pharmaceutical preparation, which indicates the high selectivity of the method. The good recoveries obtained (*Table IV*) suggest the good accuracy of the proposed method. The results showed no sign for the existence of any degradants in the sample examined.

![HPLC chromatogram](image)

*Fig. 4.* HPLC chromatogram of a 20 μL injection of 30 μg/mL TEN and 20 μg/mL EMT extracted from Truvada® tablets at 280 nm.

**Conclusions**

The work presented in this paper introduces a simple analytical procedure for the determination of TEN and EMT in binary mixture. The method is
Selective RP-HPLC DAD Method

The method is capable of resolving and simultaneously determining both TEN and EMT in a relatively short run time (< 7 min). The method is applied for quantification of the investigated compounds in their binary tablets with no interference from co-formulated additives. Therefore, the proposed method can be recommended for routine analysis and checking quality of tablets combining the two drugs.

References


Accepted by DA