HPLC Method for the Determination of Emtricitabine and Related Degradation Substances

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A new high-performance liquid chromatography method has been developed for a stability-indicating assay for emtricitabine and the quantification of its related substances. Good resolution between the peaks corresponds to process-related impurities, and degradation products from the analyte were achieved on a C18 HiQSil column using a mobile phase consisting of ammonium formate (pH 4.2) and methanol in a gradient elution mode. The detection was conducted at a wavelength of 280 nm. The investigated validation elements showed that the method has acceptable specificity, accuracy, linearity, precision, robustness and sensitivity. Detection and quantification limits were established at 0.02 and 0.05 µg/mL, respectively. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal decomposition to determine the degradation behavior. Extensive degradation was found under acid, alkaline and oxidative stress. Five related substances were consistently monitored under stress conditions. Because the method effectively separates the drug from its degradation products, it can be used as a stability indicating method and for purity control of emtricitabine.

Experimental

Instrumentation and reagents

High-performance liquid chromatography (HPLC)-grade methanol was procured from Merck (India). Ammonium formate, formic acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide were all of analytical reagent grade, procured from Merck (India). HPLC-grade water obtained from a Millipore system (Millipore, Billerica, MA) was used throughout the analysis. FTC drug substance was obtained from Cipla (Mumbai, India). Degradation impurities were isolated and purified in the lab.

HPLC instrumentation and operating conditions

A Jasco HPLC (Japan) equipped with a PU-2089 pump, a 20 µL Rheodyne 7725i manual injector and a MD-2010 diode array detector (DAD) were used. Chrompass software was connected to the detector to record the signals. A gradient LC method was used, consisting of mobile phase A: 95 volume of 0.005 M ammonium formate adjusted to pH 4.2 with formic acid and 5 volume of methanol, and mobile phase B: methanol at a flow rate of 1.0 mL/min. An HiQSil C18 column (250 × 4.6 mm) with 5 µm particle size was used for separation. The injection volume was 20 µL. The gradient conditions were as follows [time (min)/A (v/v)]: B (v/v); T0.0/95:5, T20.0/65:35, T25.0/95:5 and T30.0/95:5. The data were acquired at 280 nm for 30 min.

Isolation of degradation impurity by semi-preparative HPLC

A Shimadzu LC-8A preparative LC equipped with an SPD-10A VP, PDA detector (Shimadzu, Kyoto, Japan) was used. A Peerless Basic C18 column (250 × 15 mm, particle size 20 µ) was employed for isolation of impurities. The mobile phase consisted of water and methanol. The flow rate was kept at 4 mL/min and detection was performed at 280 nm. All force degradation impurities were isolated, and fractions collected at particular time intervals were pooled together and concentrated in a vacuum to remove methanol. The aqueous solution was lyophilized using a freeze dryer. Related substances were obtained as off-white powders and stored in a cooled airtight container. The chromatographic purities were established using HPLC–DAD and found to be above 95%.

Preparation of standard solution

A mixture of mobile phase A and methanol in the ratio of 80:20 (v/v) was used as diluent in the preparation of analytical solutions. FTC working reference standard solution (400 µg/mL) spiked with each impurity at a level of 0.5% (w/w) was used as system suitability solution. FTC working reference standard at a
concentration of 2 μg/mL and FTC drug substance at a concentration of 2,000 μg/mL were used as diluted standard solution and sample solution, respectively. A stock solution of process impurities and degradation products was prepared by first dissolving known amounts of the components in methanol and diluting to completion with the diluent.

**Stress degradation studies**
Forced degradation of FTC drug substance was conducted under acid/base hydrolysis, oxidative, thermal and photolytic stress conditions. Solutions were prepared by dissolving the drug substance in diluent and then treating with 1 M aqueous hydrochloric acid, 1 M aqueous sodium hydroxide and 30% aqueous hydrogen peroxide at 80°C for 60 min. After the degradation, these solutions were diluted with diluent and analyzed by using the proposed method. For thermal stress, a sample of drug substance was placed in a controlled temperature oven at 80°C for seven days. For photolytic stress, the sample was exposed to a photolyte of 1.2 × 10^6 lx for 120 h. After exposure to the previously discussed stress conditions, solutions of these samples were prepared by dissolving respective samples in diluent, diluting to the desired concentration and subjecting to analysis using the proposed method.

**Optimization of the chromatographic conditions**
In preliminary experiments, FTC and all its impurities were subjected to separation by reversed-phase HPLC using water, acetonitrile and methanol as organic modifiers. Generally, the selection of the column depends on the characteristics of the compounds to be separated. Depending on the solubility of FTC and its impurities, a C18 column is more preferred. The HPLC conditions were optimized to study the effects of concentration of organic modifiers, pH of buffer and gradient program on the separation of FTC and impurities.
A buffer concentration ranging from 0.005 to 0.01 M of ammonium acetate and ammonium formate was studied, and it had no effects on the retention and resolution behavior of impurities. The effect of various proportions of methanol and acetonitrile on the separation and retention behavior of all compounds was studied. No differences in retention and resolution were detected while individually using methanol or acetonitrile. Therefore, methanol was used for further optimization of the method.
Further studies were conducted on the effect of pH on resolution and retention behavior of analyte. A study was conducted ranging from pH 3.0 to 6.5. Several trials were

**Results and Discussion**

**Identification of related substances**
Based on the mass values obtained from the MS spectral data and 1H nuclear magnetic resonance (NMR) chemical shift data of isolated degradation impurities, the structures of the related substances were confirmed, as given in Figure 1. 5-Fluorocytisin (RS-1), sulphoxide (RS-2) and desamino (RS-4) impurities were identified as degradation impurities, whereas related substances lamivudine (RS-3) and salicylic acid (RS-5) were identified as process-related impurities (14).

![Figure 1. Chemical structures of FTC and related substances: FTC (A); RS-1, 5-fluorocytisin (B); RS-2, sulphoxide (C); RS-3, lamivudine (D); RS-4, desamino (E); RS-5, salicylic acid (F).](image-url)
compared, and it was observed that at pH 4.2, RS-4 was separated from FTC with good retention time and peak shapes. Finally, separation was conducted on an HiQsil C18 column maintained at room temperature at gradient elution mode. Mobile phase A consisted of 95 volume of 0.005 M ammonium formate adjusted to pH 4.2 with formic acid and 5 volume of methanol. Mobile phase B consisted of methanol, and was used at a flow rate of 1.0 mL/min with DAD detector set at 280 nm. A representative chromatogram showing the separation of each of the related substances is given in Figure 2.

![Typical chromatogram of resolution solution.](image)

Figure 2. Typical chromatogram of resolution solution.

![Chromatogram obtained after subjecting FTC to different conditions: acid hydrolysis in 1 M HCL for 60 min (A); base hydrolysis in 1 M NaOH for 60 min (B); oxidative stress in 30% H2O2 for 60 min at room temperature (C); light stress under UV light for seven days (D); dry heat for seven days at 80°C (E).](image)

Figure 3. Chromatogram obtained after subjecting FTC to different conditions: acid hydrolysis in 1 M HCL for 60 min (A); base hydrolysis in 1 M NaOH for 60 min (B); oxidative stress in 30% H2O2 for 60 min at room temperature (C); light stress under UV light for seven days (D); dry heat for seven days at 80°C (E).
Degradation behavior

Acid hydrolysis
Heating the drug in 1 M hydrochloric acid in a boiling water bath resulted in growth of the RS-1 (7%) and RS-4 (23%) peaks. RS-2 and RS-3 also generated approximately 0.1%. After heating for 60 min, the drug degraded to the extent of 30% with a corresponding increase in concentration of the degradation products (Figure 3A).

Alkaline hydrolysis
Upon heating in 1 M sodium hydroxide in a boiling water bath for 60 min, 25% degradation was achieved with a corresponding increase in concentration of the degradation products. RS-4 (24%) was observed as a major degradation product, whereas other known impurities degraded below 0.1% (Figure 3B).

Oxidative stress
The drug was found to be labile to oxidation at room temperature. It decomposed to an extent of 40% in 30% hydrogen peroxide in 15 min. The degradation increased to 80% in 60 min. The degradation product was confirmed to be RS-2 (Figure 3C).

Photolytic stress
The drug FTC was found to be stable to light stress. No considerable change was observed when the drug was exposed to ultraviolet (UV) light, both in solution and in solid state, for up to seven days (Figure 3D).

Thermal stress
FTC was found to be stable to thermal stress in a solid state. As shown in Figure 3E, no considerable change was observed after its exposure to dry heat at 80°C for seven days.

Method validation

Specificity (selectivity)
The data on degradation studies revealed that the degradation products were satisfactorily separated from the FTC and known related substances, and the peak purity data of FTC indicated that it was spectrally pure.

Linearity
The linearity of peak areas versus different concentrations was evaluated for FTC and all its related substances ranging from 0.05 to 3.0 µg/mL. The linear regression data for all tested components are presented in Table I. The relative retention factor (RRF) for each related substance was calculated with respect to FTC from the ratio of the slope of FTC to the slope of the individual related substance obtained from the regression analysis. The data shown in Table I confirmed the detector response at 280 nm to be linear over the studied ranges for all analytes.

Limits of detection and quantitation
The limit of detection (LOD) and limit of quantitation (LOQ) were determined for FTC and for each of its related substances. The LOD and LOQ values for related substances were found to be 0.02 and 0.05 µg/mL, respectively. The calculated LOD and LOQ levels of all the components were verified for precision. Relative standard deviation (RSD) was in the range of 4.71–8.91% for LOD and 2.57–7.12% for LOQ, respectively. The results are reported in Table I.

Precision
The precision of the method was studied for repeatability and intermediate precision. Repeatability was demonstrated by analyzing six separate FTC sample solutions that were prepared by spiking the related substances at specific levels. The RSD (0.96–1.81%, n = 6) was evaluated for each related substance. The intermediate precision of the method was determined on six separate sample solutions prepared from the same lot by spiking the related substances at specific levels by a different analyst using a different mobile phase and on a different day with a different lot of the same brand of column. The overall RSD was evaluated and found to be in the range of 0.97–1.81% for all related substances, which was within the acceptance criterion of not more than (NMT) 10.0% RSD. The results are presented in Table II.
**Accuracy (recovery)**

The accuracy of the method for all related substances was determined by analyzing FTC sample solutions spiked with all of the related substances at three different concentration levels of LOQ, 100 and 150% of each, in triplicate at the specified limit. The recovery of these related substances was found to be between the predefined acceptance criteria of 90.0–110.0%, and the results are given in Table III.

**Robustness**

To evaluate the robustness of the method, the influence of small and premeditated alterations of analytical parameters on the quantification of the related substances and selectivity was studied. The selected parameters were mobile phase composition (+/- 2% of gradient composition), pH of the mobile phase (+/- 0.5 units), flow rate (+/- 20%) and wavelength (+/- 2 nm).

Only one parameter was changed while the others were kept unaltered. The mean and RSD for each related substance were evaluated. The difference between the mean values (for all related substances from each of the robustness parameters) from the repeatability mean results was found to be below 10.0%. The results are presented in Table IV. The studies indicated no effect on the determination of related substances or the selectivity. Therefore, the test method is robust for the quantification of related substances.

**Application of method**

FTC drug product (Emtriva) was analyzed using the proposed method in triplicate. The results showed the presence of RS-1, RS-2, RS-3, RS-4 and RS-5 in the range of 0.05–0.11%. RSD (n = 3) for the related substances present in the samples was below 10.0%. The results are presented in Table V.

**Conclusion**

A highly sensitive and specific LC method was proposed for the determination of related substances in FTC. The method is more highly sensitive and selective toward the determination of related substances than previously reported methods by Seshachalam et al. (12) and Ashenafi et al. (13). Because all related substances are satisfactorily separated and can be identified based on RRT, no further degradation of the sample is required to prepare a system suitability test solution, as reported by Ashenafi et al. Five related substances were identified and determined by this method using quantitation factor to accurately determine the impurities. The stress degradation study shows that FTC is a labile molecule when stressed with...
acid, alkali and oxidation. A stability-indicating method was developed that separates the entire degradation product formed under a variety of conditions, and better separation was achieved than in previously reported methods. This method proved to be simple, accurate, specific and selective. Hence, it is recommended for analysis of the drug and related substances by the pharmaceutical industry.

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