Stability-Indicating HPLC Method for Determination of Fosamprenavir Calcium

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A novel stability-indicating reverse-phase high-performance liquid chromatographic (HPLC) method has been developed for quantitative determination of Fosamprenavir Calcium, HIV-1 protease inhibitor. Chromatographic separation was achieved using an YMC Pack ODS AQ (150 mm × 4.6 mm × 3.0 μm) HPLC column in isocratic mode employing 0.05 M Potassium dihydrogen orthophosphate monohydrate (pH 6.8) buffer and Acetonitrile in the ratio 60:40 (v/v) with a flow rate of 0.8 mL min⁻¹. Detector wavelength was monitored at 265 nm and column temperature was maintained at 40°C. Fosamprenavir calcium was exposed to thermal, photocytic, humidity, water, acid, base and oxidative stress conditions. Considerable degradation of the drug substance was found to occur under acid, base and oxidative stress conditions. Peak homogeneity data of Fosamprenavir Calcium obtained by photodiode array detection demonstrated the specificity of the method in the presence of degradants. The degradation products were well resolved from the main peak of Fosamprenavir, indicating that the method is specific and stability-indicating. The HPLC method was validated as per International Conference on Harmonization guidelines with respect to specificity, precision, linearity, accuracy and robustness. Regression analysis showed a correlation coefficient value greater than 0.999. The accuracy of the method was established based on the recovery obtained for Fosamprenavir Calcium.

Introduction

During the past decade, human immunodeficiency virus (HIV) infection has become a largely manageable but incurable disease. This is due to the advent of highly active antiretroviral therapy, in which patients are treated with a cocktail of drugs designed to reduce their viral loads to extremely low levels (1).

More than 60 million people have been infected with HIV, known as cause of the acquired immunodeficiency syndrome (AIDS). HIV/AIDS is now the leading cause of death in sub Saharan Africa and is the fourth biggest killer world wide.

HIV is able to evade immunological pressure, to adapt to a variety of cell types and growth conditions and to develop resistance against currently available drug therapies, which include nucleoside reverse-transcriptase inhibitors, non-nucleoside reverse transcriptase, nucleotide reverse transcriptase inhibitors, HIV-1 protease inhibitors (PIs), fusion inhibitors and the more recent CCR5 and integrase inhibitors.

Fosamprenavir Calcium, (1S2R)-3-[(4-aminophenyl)sulfonyl]-2-methylpropyl]amino]-1-(phenylmethyl)-2{(phosphonoxy) propyl]carboxylic acid C-(3S)-tetrahydro-3-furanylester calcium salt (Figure 1), is the phosphate ester prodrug of HIV PI amprenavir (2–4). Fosamprenavir was first approved by the Food and Drug Administration in 2003 (5) and then by the European Medicines Agency in 2004 (6). It is presented either as coated tablets or as oral suspension and it was developed to overcome adherence barriers with amprenavir formulations, such as pill size and burden, food and water restrictions. According to the Biopharmaceutics Classification system, fosamprenavir is classified as class II (7). Currently, Fosamprenavir Calcium is available in the market under the brand name of LEXIVA, formerly known as GXLL. It is administered two times a day (700 mg).

Each tablet also contains the inactive ingredients colloidal silicon dioxide, croscarmellose sodium, magnesium stearate, microcrystalline cellulose, and povidone. The tablet film coating, LEXIVA pink (700 mg dose), contains hypromellose, iron oxide red, titanium dioxide and triacetin.

A dissolution method has appeared in the literature for the discriminating method of dissolution for Fosamprenavir tablets (8). The literature reported the electrochemical evaluation and determination of antiretroviral drug fosamprenavir using boron-doped diamond and glassy carbon electrodes (9). However, extensive survey revealed no stability-indicating HPLC/UPLC methods for quantitative determination of Fosamprenavir Calcium in bulk active pharmaceutical ingredient. Further, no official or draft monograph on Fosamprenavir Calcium was published in any of the pharmacopoeia for compendial applications.

Therefore, it was felt necessary to develop an accurate, rapid, specific and stability-indicating method for the determination of assay of Fosamprenavir Calcium. This method is also used for the quantitative analysis of Tablet Dosage forms. In the present developed method, there are no interferences observed from blank and excipients at Fosamprenavir peak retention. The main advantages of this method are its simplicity and accuracy with shorter run time.

Materials and Methods

Materials

Fosamprenavir Calcium reference standard and test samples were received from the Analytical Research and Development department of Hetero Research Foundation (Hyderabad, India). The related compounds, i.e., Impurity-A and Impurity-B, were also received from the synthetic division of Hetero Research Foundation. Impurity-A is a degradation impurity and Impurity-B (amprenavir) is a process-related impurity. HPLC grade Acetonitrile was purchased from Merck, Darmstadt, Germany. Analytical reagent grade Potassium dihydrogen orthophosphate monohydrate was purchased from Rankem, Mumbai, India. High-pure water was prepared by using a Millipore Milli ‘Q’ plus purification system.

Equipments

The HPLC system used for initial chromatographic development was a Waters alliance HPLC (Milford, MA) 2695 separation
module equipped with quaternary gradient pumps, an inbuilt auto injector, a 270852 thermostatic compartment and a 2487 UV detector. Empower chromatography manager software was used for data acquisition and system suitability calculations. A photodiode array (PDA) detector was used for determining peak purity. Photo stability studies are carried out in a photostability chamber (Atlas Suntest CPS+). Thermal stability studies are carried out in a dry hot air oven (Cintex precision hot air oven).

**Chromatographic conditions**

The chromatographic separation was achieved on an YMC Pack ODS AQ column, 150 × 4.6 mm, 3.0 μm (YMC Co., Ltd). The mobile-phase composition was pH 6.8 ± 0.05 buffer (6.8 g of Potassium dihydrogen orthophosphate monohydrate in 1 000 mL of water and adjusted to pH 6.8 ± 0.05 with triethylamine solution) and Acetonitrile in the ratio 60:40 (v/v). The mobile phase was filtered and degassed through 0.22 μm filter paper. The flow rate of the mobile phase was kept at 0.8 mL min⁻¹. The column temperature was maintained at 40 °C and the detector wavelength was monitored at 265 nm. The injection volume was 10 μL. Methanol and Acetonitrile in the ratio 90:10 (v/v) was used as diluent. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

**Preparation of standard solutions**

Two milligrams of the Fosamprenavir Calcium reference standard were placed in a 10-mL volumetric flask, dissolved and diluted to the mark with diluent. Working solution of (0.2 mg/mL) test solution was prepared by dissolving an appropriate amount of test in the diluent. A stock solution of impurity mixture (A and B) at 0.02 mg/mL was also prepared in diluent for specificity.

**Preparation tablets**

The coated layer was removed from an adequate number of Lexiva tablets (700 mg Fosamprenavir) with a knife and thoroughly

![Chemical structure and name](image-url)
ground to a fine powder with mortar and pestle. The powder was transferred into a 100-mL volumetric flask to obtain a 1.0-mg/mL concentration of Fosamprenavir and sonicated for 20–25 min. The resulting stock solution was used for further dilutions. The resulting solution was filtered through a 0.22-μm pore size Nylon 66 membrane filter.

Method validation
The stability-indicating reverse-phase method developed was validated according to current guidelines (10, 11). Specificity, precision, linearity, accuracy, robustness, test solution stability and mobile-phase stability were evaluated.

Specificity
The specificity of the developed HPLC method for Fosamprenavir Calcium was determined in the presence of its impurities and degradation products. Forced degradation studies were also performed on Fosamprenavir Calcium to provide an indication of the stability-indicating property and specificity of the proposed method. The stress conditions employed for degradation study included: light, 1.2 million lux h (conducted as per International Conference on Harmonization, ICH Q1B option-1), thermal (105°C, 7 days), humidity (25°C, 90% relative humidity, 7 days), acid hydrolysis (2.0 N HCl, 12 h of heating at 80°C), base hydrolysis (2.0 N NaOH, 12 h of heating at 80°C), water hydrolysis (8 h of heating at 80°C) and oxidation (6% H2O2, 8 h of heating at 80°C).

The photodegradation was conducted by exposing the Fosamprenavir Calcium sample in solid state to light, providing an overall illumination of not less than 1.2 million lux h and an integrated near-ultraviolet energy of not less than 200 Wh/m² (12), which lasted approximately about 2 days in the photostability chamber. The generated stressed samples of Fosamprenavir Calcium generated were checked for peak purity by using the Waters PDA detector. The purity angle is within the purity threshold limit obtained in all stressed samples, which demonstrates the peak homogeneity of the analyte. Assay studies were conducted for stress samples against qualified reference standards and the mass balance [% assay of Fosamprenavir Calcium + % of total impurities] was calculated. The assay was also calculated for bulk samples by spiking impurities (A and B) at specification level (i.e., 0.15% with respect to analyte concentration 0.2 mg/mL). A typical HPLC assay chromatogram of Fosamprenavir Calcium and test spiked with impurities is shown in Figure 2.

Precision
The system precision of the assay method was evaluated by conducting six replicate injections of Fosamprenavir Calcium standard solution. The percentage of relative standard deviation (RSD) was calculated for the area of the Fosamprenavir Calcium peak from six replicate injections. The method precision of the assay method was evaluated by conducting six independent assays of test sample of Fosamprenavir Calcium against qualified reference standard. RSD was calculated for six assay values obtained. The intermediate precision of the method was also evaluated with a different analyst and a different instrument in the same laboratory.

Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample. Linearity test solutions for the assay method were prepared from a stock solution at five concentration levels ranging from 80 to 120% of the assay analyte concentration (80, 90, 100, 110 and 120%). Regression analysis was performed by least squares using the peak area versus concentration data.

Accuracy/recovery
Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of Fosamprenavir Calcium. The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e., 80, 100 and 120% of the analyte concentration (0.2 mg/mL), in bulk drug sample and the percentage recoveries were estimated.

Robustness
To determine the robustness of the developed assay method, experimental conditions were purposely altered and the assay content of the Fosamprenavir Calcium was evaluated. To study the effect of flow rate on the assay, it was changed to 0.6 to 1.0 mL/min. The effect of the percent organic strength on assay was studied by varying by ±5.0%. The effect of pH was studied at 6.6 and 7.0 instead of 6.8. The effect of column temperature was studied at 38°C and 42°C instead of 40°C. In all of the varied conditions, the components of the mobile phase were held constant, as stated previously. In all the deliberately varied chromatographic conditions, the selectivity and the performance of the method were unchanged, which proves the robustness of the method.

Test solution stability and mobile-phase stability
The test solution stability of Fosamprenavir Calcium for the assay method was conducted by keeping both the test solution and reference standard in tightly capped volumetric flasks at room temperature for 12 and 24 h. The same sample solutions were assayed at 6 h intervals throughout the study period. The mobile-phase stability was also conducted by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6 h interval up to 24 h. The prepared mobile phase was kept constant during the study period. The percentage recovery of assay of Fosamprenavir Calcium was calculated for the study period during mobile phase and solution stability experiments.

Results and Discussions
Method development and optimization
The primary criteria for the development of a successful HPLC method for the determination of assay of Fosamprenavir Calcium was that the method should be able to separate impurities and degradants within shorter run time and should be accurate, reproducible, robust and stability indicating.

The primary objective of the chromatographic method was to achieve the separation of Impurity-A and Impurity-B from Fosamprenavir Calcium by using different stationary phases like C18, C8 and cyan and different mobile phases containing buffers like acetate and phosphate with different pH (5–7) and using organic modifiers like acetonitrile and methanol in the mobile phase (Table I). The chromatographic separation was achieved on...
YMC Pack ODS AQ 150 × 4.6 mm, 3.0 μm column. The system suitability parameters considered for Fosamprenavir Calcium are repeatability, tailing factor and theoretical plates. The tailing factor of the Fosamprenavir Calcium is 1.4, and theoretical plates (column efficiency) were 8562. The percent RSD of Fosamprenavir peak from five replicate injections was found to be 0.2.
The developed method is specific to Fosamprenavir Calcium and its two impurities, i.e., Impurity-A [3-amino-1-(4-amino-N-isobutylphenylsulfonamido)-4-phenylbutan-2-yl dihydrogen phosphate] and Impurity-B [(3S)-oxolan-3-yl-N-[(2S,3R)-3-hydroxy-4-[N-(2-methyl propyl)(4-aminobenzene)sulfonamido]-1-phenylbutan-2-yl]carbamate]. The chemical structures of Fosamprenavir Calcium, Impurity-A and Impurity-B are shown in Figure 1.

**Method validation results**

**Specificity**
Specificity is the ability of the method to measure the response of the analyte in the presence of its potential impurities (13, 14). Stress testing of the drug substance can help identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule, and to validate the stability-indicating power of the analytical procedures. No peak due to blank and degradation products detected at the retention time of Fosamprenavir Calcium. All known impurities are separated from Fosamprenavir peak.

**Precision**
RSD for system precision and method precision studies for the assay of Fosamprenavir Calcium was found to be within 1.0. The percent assay of six individual test preparations of Fosamprenavir Calcium ranged from 99.3 to 100.1% with an RSD of 0.18%. The percent assay of six individual test preparations of Fosamprenavir Calcium in the intermediate precision study ranged from 99.4 to 100.2% with an RSD of 0.31%, thus confirming good precision of the method.

**Linearity**
The plot of peak area of Fosamprenavir versus concentration ranging from 0.16 to 0.24 mg/mL was linear, and the data were

![Figure 3. HPLC chromatograms of Fosamprenavir Calcium degradation in: (A) 2.0 N HCl (t = 12 h), (B) 2.0 N NaOH (t = 12 h) and (C) 6% H2O2 (t = 8 h).](image-url)
subjected to statistical analysis using a linear regression analysis with least squares. The linear regression equation and correlation coefficient were \( y = 22.820 + 816x - 50,549, 0.999 \), respectively. RSD values for the peak areas of Fosamprenavir at each level are within 1.0. These results show that an excellent correlation existed between the peak area and concentration of the analyte. Residuals were within \( \pm 2\% \) scattered with respect to 100% concentration response. Sensitivities were scattered within \( \pm 2\% \) with respect to 100% concentration sensitivity.

**Accuracy and recovery**
The accuracy of the assay method was evaluated in triplicate at three concentration levels 0.16, 0.20 and 0.24 mg/mL, with respect to specification level, which is 0.20 mg/mL. The same procedure was adopted for tablet powder. The percentage recovery of Fosamprenavir Calcium found in bulk drug sample and tablet dosage forms was found in the ranges of 99.11–100.42% and 98.83–100.21%, respectively.

**Robustness**
No significant change in the assay value was observed for any of the deliberately varied chromatographic conditions. The retention time of the Fosamprenavir Calcium was slightly shifted, i.e., 3.8–4.0 min, when using a different lot of the same brand column. The tailing factor and theoretical plates of the Fosamprenavir Calcium were not much affected by new lot column. The percent RSD of Fosamprenavir peak from five replicate injections was found to be 0.26. The mean percent assay of Fosamprenavir Calcium was found to be between 99.6 and 100.0% from robustness studies involving deliberate changes in flow rate, pH of the mobile phase, mobile-phase composition and column temperature. The system suitability parameters like tailing factor (1.25–1.42) and the RSD values are well within the limits, which confirm the robustness of the developed method.

**Test solution stability and mobile-phase stability**
The RSD of the assay of Fosamprenavir Calcium during solution stability and mobile-phase stability experiments was within 1.0%. No significant changes were observed in the content of the assay of Fosamprenavir Calcium during solution stability and mobile-phase stability experiments. The solution stability and mobile-phase stability experiment data confirm that the sample solutions and the mobile phase are stable up to 12 h.

### Results of Forced Degradation Studies

All forced degradation samples were analyzed at 1.0 mg/mL concentration of Fosamprenavir Calcium using the PDA detector to ensure the homogeneity and purity of Fosamprenavir Calcium peak. Significant degradation was observed in acid hydrolysis (2.0 N HCl at 80°C for 12 h), base hydrolysis (2.0 N NaOH at 80°C for 12 h) and oxidation (6% H₂O₂ at 80°C for 8 h) (Figure 3). Base hydrolysis and oxidation led to the formation of Impurity-A, while Impurity-A and Impurity-B formed in acid hydrolysis. This was confirmed by co-injecting Impurity-A and Impurity-B to these degradation samples. The impurities are well separated from each other in base, acid and oxidation degradation samples (Table II). Fosamprenavir Calcium was found stable under water hydrolysis (water at 80°C for 8 h), thermal degradation (105°C for 7 days), humidity (25°C, 90% RH for 7 days) and photolytic (exposed to 1.2 million lux h visible light and 200 W h/m² UV light) degradation conditions. The purity of Fosamprenavir Calcium was unaffected by the presence of its impurities and degradation products which were well separated from the Fosamprenavir Calcium peak and thus confirms the stability-indicating power of the developed method. The percent assay and mass balance (% assay of Fosamprenavir Calcium + % of total impurities) of the all degraded samples varied between 86.3 and 99.8 and 98.9 and 100.2%, respectively. The forced degradation results and mass balance results are shown in Table III.

### Conclusions
The newly developed RP-HPLC isocratic method for the determination of Fosamprenavir Calcium assay in bulk active pharmaceutical ingredients and tablet dosage forms was found to be specific, precise, accurate and robust. The stability-indicating nature of the proposed method was established by performing forced degradation studies, which provided the degradation behavior of Fosamprenavir Calcium under various conditions. The method validation data showed satisfactory results for all tested method parameters. Hence, the developed HPLC method can be used for routine analysis of production samples and for stability of bulk samples of Fosamprenavir Calcium.

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