METHOD DEVELOPMENT AND METHOD VALIDATION OF TENOFOVIR IN HUMAN K3 EDTA PLASMA BY LC-MS/MS

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ABSTRACT
Currently there is a need in the pharmaceutical environment to develop bio analytical methods for the determination of Tenofovir in human plasma. The aim of this study is to develop a suitable, highly specific, and sensitive bioanalytical method for the quantitation of Tenofovir in the low nano gram range in human plasma. The main objective of this work is to develop rapid, selective and sensitive LC-MS/MS method that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time.

Keywords: Tenofovir, human plasma, LC-MS/MS System, validation.

INTRODUCTION
Tenofovir is an example of antiretroviral drug/ nucleotide analogue reverse transcriptase inhibitors. The chemical structure of tenofovir is

Chemically tenofovir is a 1-(6-aminopurin-9-yl) propan-2- yloxy methyl phosphonic acid. Tenofovir is a nucleotide analogue of adenosine monophosphate\(^1\). Its active metabolite, tenofovir diphosphate, competes with natural deoxyadenosine triphosphate for the active binding site on the HIV-induced reverse transcriptase (HIV DNA polymerase)\(^{1,2,3}\).

Fluconazole is an example of antifuangal angent and is used as an internal standard (IS). The chemical structure of fluconazole is
Chemically fluconazole is 2,4-difluoro-α₁,α₁-bis(1H-1,2,4-triazol-1-ylmethyl) benzyl alcohol. Fluconazole is a highly selective inhibitor of fungal cytochrome P-450 dependent enzyme lanosterol 14-α-demethylase. This enzyme functions to convert lanosterol to ergosterol. The subsequent loss of normal sterols correlates with the accumulation of 14-α-methyl sterols in fungi and may be responsible for the fungistatic activity of fluconazole. Mammalian cell demethylation is much less sensitive to fluconazole inhibition[2,3,4].

MATERIALS AND METHODS

Instrumentation

An API 3200 LC/MS/MS system equipped with a pump (Shimadzu LC-20ADvp), an auto sampler (Shimadzu SIL-HTc), analytical column Kromosil 100, C18, 5µ (150mm×4.6mm)(flexit, Germany) and data acquisition system and quantitation program (Applied Biosystems Analyst® Software version 1.4.2) was used for the determination of Tenofovir in human plasma. The interface used with the API 3200 LC/MS/MS was a Turbo ionspray®. The positive ions were measured in MRM mode for the two analytes and the internal standards.

Tenofovir stock solution preparation

Tenofovir Working standard was weighed accurately equivalent to 10mg and it was transferred into 10.000mL volumetric flask. It was dissolved in about 5.000mL of Water and made up to 10.000 mL with the same to get 1.000mg/mL.

Internal standard (Fluconazole) stock solution preparation

Fluconazole working standard was weighed accurately equivalent to 10mg and transfer it into 10.000mL volumetric flask, It was dissolved in about 5.000mL of Methanol and made up to 10.000 mL with the same to get 1.000mg/mL.

Preparation of plasma sample solution
500µL of plasma sample was taken and 50µl of ISTD dilution (0.400 µg/mL Fluconazole) was added. 500µl of 2% of ortho phosphoric acid in water was also added to it and vortexed. Then it was kept aside for Solid phase extraction.

2 ml of methanol followed by 2ml of 0.5% of Ortho phosphoric acid in water was added for Conditioning and Equilibrium of the catridge.1050µL of spiked plasma sample was loaded. 1mL of milli-Q-Water and 1 ml of 2% of Ortho phosphoric acid followed by 1 mL of Water was added to wash the sample. The cartridges were dried for 2 minutes. Then the samples were eluted with 1 ml of 5% ammoniated methanol. The sample was evaporated to complete dryness under stream of Nitrogen gas at ≤50°C. The residue was reconstituted with 1.000 mL of reconstitution solution(Methanol:2.5mM Ammonium acetate pH 6.0). Reconstituted sample was loaded into the HPLC vial and 25µl of sample was injected into LC-MS/MS system.

Method development

Chromatographic mode: LC/MS/MS-API-3200
Mobile phase: 2.5 mM Ammonium acetate (w/v) buffer solution : Methanol (30:70)
Buffer: 2.5 mM Ammonium acetate (w/v) buffer solution
Column: Kromasil100, C18, 5µ, 150*4.6 mm
Isocratic/gradient mode: Isocratic
Auto sample temperature: 15°C
Syringe speed: 5µl/sec
Rinsing volume: 600µl
Column temperature: 40°C
Injection volume: 25µl
Retention time
Tenofovir: 1.50min
Fluconazole: 2.25min
Detection parameters

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Tenofovir</th>
<th>Fluconazole</th>
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<tbody>
<tr>
<td>Parent mass</td>
<td>288.10</td>
<td>307.10</td>
</tr>
<tr>
<td>Product mass</td>
<td>176.10</td>
<td>220.30</td>
</tr>
</tbody>
</table>
Validation parameters

System Suitability
System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture equivalent to MQC1 concentration of the calibration curve.

Linearity
The linearity of the method was determined by using a $1/x^2$ weighted least square regression analysis of standard plots associated with a ten-point standard curve.

Recovery
Recovery for Analyte
The percentage mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1, MQC2 and LQC levels.

Recovery for Internal Standard
The percentage mean recoveries were determined by measuring the responses of internal standard in the extracted samples against unextracted samples respectively.

Precision
The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability.

Accuracy
The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. At a minimum, three concentrations representing the entire range of the
standard curve should be studied: one within the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

RESULTS

Calibration curve of tenofovir

<table>
<thead>
<tr>
<th>Linearity</th>
<th>( r \geq 0.9962 )</th>
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<tbody>
<tr>
<td>Calibration curve range</td>
<td>5.01 to 499.87 ng/mL.</td>
</tr>
<tr>
<td>Specificity</td>
<td>No significant interferences observed at tested matrices for Tenofovir and IS.</td>
</tr>
<tr>
<td>Intra Batch Accuracy</td>
<td>98.16% to 105.39%.</td>
</tr>
<tr>
<td>Intra Batch Precision</td>
<td>5.66% to 8.39%.</td>
</tr>
<tr>
<td>Inter Batch Accuracy</td>
<td>99.23% to 105.06%.</td>
</tr>
<tr>
<td>Inter Batch Precision</td>
<td>3.93% to 7.41%.</td>
</tr>
<tr>
<td>Mean Recovery of Analyte across QC level</td>
<td>81.39%.</td>
</tr>
<tr>
<td>Mean Recovery of Analyte at LQC, MQC, HQC level</td>
<td>84.18, 77.48, 82.52 % respectively</td>
</tr>
<tr>
<td>Mean % CV recovery of Analyte across QC level</td>
<td>4.28%.</td>
</tr>
<tr>
<td>Recovery of Internal standard (Fluconazole)</td>
<td>75.27%</td>
</tr>
<tr>
<td>Mean % CV recovery of Internal standard</td>
<td>4.43%</td>
</tr>
</tbody>
</table>

REFERENCES

2. www.medicinenet.com
3. www.drugbank.com

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