Chapter 6

DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE ANALYSIS OF 2-ISOPROPYL-5-METHYLCYCLOHEXYL DIHYDROXY ACETATE, IN LAMIVIDINE
6.1. INTRODUCTION

Lamivudine (LAM) is chemically (2R, cis) - 4-amino-1(2-hydroxymethyl-1,3-oxathiolan-5yl)-(1H)pyrimidin-2-one [1]. Lamivudine is (-) enantiomer of 2′-deoxy-3′-thiacytidine [2], is a cytosine analog with potent activity against human immunodeficiency (HIV) and hepatitis B viruses (HBV) through inhibition of reversed transcriptase activity. LAM is a (-) enantiomer of the racemic mixture which shows much less cytotoxicity than (+) enantiomer. LAM is used in the treatment of HBV infections and it has strongly recommended for the treatment of HIV infections in combination with other antiviral drugs [3].

Lamivudine [4-6] is soluble in water, sparingly soluble in methanol, slightly soluble in ethanol. Moreover, lamivudine is active against zidovudine-resistant HIV [7]. The US Department of Health and Human Services’ current guideline for the treatment of established HIV infection strongly recommends LAM in combination with another nucleoside reverse transcriptase inhibitor and either a protease inhibitor or efavirenz [8]. The usual dosage of LAM is 150 mg twice daily or 300 mg once daily in combination with other antiretroviral agents [9].

Pharmaceutical genotoxic impurities (PGIs) may induce genetic mutations, chromosomal breaks (rearrangements) and they have potential to cause cancer in human [10-11]. Therefore exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicological importance [12]. Hence it is significant for process chemists to avoid such genotoxic impurities in the manufacturing process [13]. However it would be difficult or impossible to eliminate PGIs completely from the synthetic scheme. Therefore it is a great challenge to analytical chemists to develop an appropriate analytical method to quantify the impurity accurately and control their levels in APIs. According to the European Medicines Evaluation Agency (EMEA) and feedback from US Food and Drug Administration (USFDA) the proposed use of a threshold of toxicological concern (TTC), it is accepted that genotoxic impurities will be limited to a daily dose of 1.0-1.5 µg/day [14-15].
Several analytical methods have been developed for the determination of LAM either individually or in combination with other anti-retroviral drugs in the dosage forms and in biological fluids. Examples of these methods are spectrophotometric [16-29], high performance liquid chromatographic (HPLC) [30-39]. To the best of our knowledge no published method is available for the simultaneous determination of 2-Isopropyl-5-Methylcyclohexylidihydroxyacetate and Lamivudine API using LC-MS/MS. This method provides high degree of precision, accuracy, sensitivity and stability by simple liquid – liquid extraction based on liquid chromatography separation and detection in positive ion mode by electro-spray tandem mass spectrometry.

The present study was undertaken to develop a sensitive and rapid LC/MS/MS method for the determination of 2-Isopropyl-5-Methylcyclohexylidihydroxyacetate a genotoxic impurity in Lamivudine API. Due to its higher selectivity and sensitivity LC/MS/MS has been adopted for quantification of 2-Isopropyl-5-Methylcyclohexylidihydroxyacetate in Lamivudine is used in the treatment of HBV infections and it has strongly recommended for the treatment of HIV infections in combination with other antiviral drugs.

Structure of Lamivudine and impurity:

6.2. Structure of Lamivudine:

Chemical formula: C₈H₁₁N₃O₃S
Molecular weight: 229.25
6.3. Structure of 2-Isopropyl-5-Methyl cyclohexyl dihydroxyacetate:

![Chemical structure of 2-Isopropyl-5-Methyl cyclohexyl dihydroxyacetate]

Chemical formula: \( C_{12}H_{22}O_4 \)
Molecular weight: 230.30

EXPERIMENTAL

6.4. Chromatographic Conditions:

Chromatographic conditions adopted in the present study are as follow:

- **Column**: Symmetry c-18, 150X4.6mm, 3.5μm
- **Flow rate**: 0.8 mL/min
- **Injection volume**: 15μL
- **Sample conc**: 10.0 mg/mL
- **Diluent**: Water : Acetonitrile (30:70 v/v)
- **Column temperature**: 35°C
- **Run time**: 12 minutes

6.5. MATERIALS AND METHODS

6.5.1. Chemicals and Reagents

Acetonitrile of HPLC grade was purchased from Merck (Mumbai, India). Analytical grade Formic acid, HPLC grade water was purchased from Merck, (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Reference
substance of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate was obtained from local manufacturing unit in Hyderabad.

6.5.2. Preparation of stock and standard solutions

Primary stock solutions of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate was prepared in 10 mg/mL of impurities in 100 ml of diluent. Further dilution 0.001 mg/mL with diluents to get 0.000009 mg/mL. Diluted final concentration 9 ppm is used to get working solutions for obtaining calibration curve.

6.5.3. HPLC operating conditions

A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to inject 15 μL aliquots of the processed samples on a Symmetry C-18, 150X4.6mm, 3.5μm, which was kept at 35 ± 2°C temperature. The mobile phase, a mixture of 1.0 ml formic acid: acetonitrile (60:40 v/v) was filtered through 0.45 μm membrane filter (Millipore, USA or equivalent), then degassed ultrasonically for 12 min and delivered at a flow rate of 0.8 ml/min into the mass spectrometer electrospray ionization chamber.

6.5.4. Mass spectrometry operating conditions

Quantitation was achieved with MS-MS detection using a Applied bio system (AB SCIEX) API-4000 mass spectrometer (Foster City, CA, USA) equipped with turboionspray™ interface at 420°C. The MS/MS method operated at positive mode. The ion spray voltage was set at 4500 V. The source parameters viz., the ion source gases GS1, GS2 and nebulizer gas were set at 30, 28, 14 psi respectively. The compound parameter viz. the declustering potential (DP) and entrance potential were set at 55 and 10 V. In this method 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate was monitored with its molecular ion [M+H]+ m/z 231.2 (protonated) and Lamivudine was monitored with its molecular ion [M+H]+ m/z 230.2 (protonated). The analytical data obtained were processed by Analyst software™ (version 1.5.1).
6.6. RESULTS AND DISCUSSION

6.6.1. Method development and optimization

Optimization of chromatographic conditions was performed, particularly the composition of mobile phase, through several trials to achieve symmetric peak shapes of the analytes peaks, as well as short run time and low cost. Positive resolution mode of lamivudine was achieved by using acetonitrile as an organic content in the mobile phase. Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C18 and C8 of different makes like Hypersil BDS column, Inertsil ODS Column and symmetry C18 column. Finally symmetry C18 column was found to give the best chromatographic resolution with a flow rate of 0.8 mL/min and total run time of 15 mins. The 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate and Lamivudine were eluted at 7.97 and 5.1 min with selective ion monitoring (SIM) mode. The inclusion of 1mL Formic acid instead of pure water enhanced the response and improved the reproducibility.

6.7. Method validation

6.7.1. Specificity and selectivity

Specificity is the ability of the method to assess unequivocally the analyte response in presence of components that may be expected to be present in the sample. Lamivudine and 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate compounds solutions were prepared individually at a concentration of about 0.01mg/mL in the diluents and a solution of Lamivudine spiked with 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate were also prepared. Specificity was established by injecting Lamivudine spiked sample with its impurity where in no interference was observed. Blank and specificity chromatograms are shown in Fig. 6.1 (a), (b).
Fig 6.1. (a) Blank chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate

Fig 6.1(b) Specificity chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate
Robustness

The robustness of the developed method was studied with slight and deliberate changes in experimental conditions. The effect of changes in flow rate of mobile phase (-2% to +2%) while the amounts of the other mobile phase components were held constant, column oven temperature (-2°C to +2°C), i.e., at 33°C and 37°C, buffer units were held constant.

6.7.2. Determination of LOD and LOQ

The LOD and LOQ, as a measure of method sensitivity, were calculated from S/N (signal to noise) ratios. To determine LOD and LOQ values for a 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate concentration were reduced sequentially such that they yield S/N ratio as 3.2 and 10.1 respectively. The determined LOD and LOQ chromatograms were shown in Fig 6.2(a),(b). Data generated from six injections of (without API) containing 9 ppm of each 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate with respect to an API sample concentration 10 mg/mL. The LOQ of 9 ppm is typical for the 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate, with a LOD approximately three times less than LOQ. In addition, the relative efficiency of SIM modes in sensitivity improvement was also evaluated.
Fig 6.2. (a) LOD chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate

Fig 6.2 (b) LOQ chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate
6.7.3. Recovery studies

The recovery studies by the standard addition method were performed to evaluate accuracy and specificity, accordingly the accuracy of the method was determined in triplicate at LOQ level in bulk drug sample. The recoveries were calculated. Excellent recovery values of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate 101.75 – 99.59 percentage were obtained. At such a low levels these recoveries and %RSD is <1.0 was satisfactory. Sample and Spiked at LOQ chromatograms are shown in Fig 6.3(a),(b) and the relative standard deviation, %RSD were calculated from the average of triplicate analysis, which were shown in Table1. Further, the stability of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate was found as 48 hr and the stability of this impurity at different time intervals is presented in Table 2.

![Fig6.3. (a) Sample chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate](image-url)
Fig 6.3 (b) Spiked chromatogram of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate

Table 1: Accuracy/recovery of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound name</th>
<th>Sample area</th>
<th>Level</th>
<th>Standard area</th>
<th>Spiked area</th>
<th>Theoretical concentration</th>
<th>Measured concentration</th>
<th>%Recovery</th>
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<tbody>
<tr>
<td>1</td>
<td>2-Isopropyl-5-Methylcyclohexyl dihydroxyacetate</td>
<td>LOQ</td>
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<td>98817</td>
<td>100545</td>
<td>9</td>
<td>9.1574</td>
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<tr>
<td></td>
<td></td>
<td>50</td>
<td>0</td>
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<td></td>
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<td>1472410</td>
<td>1466322</td>
<td>135</td>
<td>134.4418</td>
<td>99.59</td>
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Table 2: Solution stability data of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate in diluents

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound name</th>
<th>Injection time (hr)</th>
<th>Sample area</th>
<th>Standard area</th>
<th>Spiked area</th>
<th>Theoretical concentration</th>
<th>Measured concentration</th>
<th>%Recovery</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2-Isopropyl-5-Methylcyclohexyl dihydroxyacetate</td>
<td>0</td>
<td>0</td>
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<td>97840</td>
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<td></td>
<td>48</td>
<td>0</td>
<td>99777</td>
<td>99645</td>
<td>9</td>
<td>8.9881</td>
<td>99.87</td>
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6.7.4. Linearity and range

The linearity test for the method was performed according to the guidelines laid by ICH. This method was evaluated at six different concentrations of analytes with in the range of 9-108 ng/mL. These standard solutions were prepared by suitable dilution of stock solution with mobile phase. The linearity of the plot was evaluated using least squares linear regression analysis by selective ion monitoring (SIM). The linearity of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate was satisfactorily established with a six point calibration curve between LOQ and 150% of analyte concentrations (40%, 60%, 80%, 100%, 120% and 150%). The calibration curve was produced by plotting the average of triplicate of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate injections against the concentrations expressed in percentage. The slope, intercept and correlation coefficient values were derived from linear least-square regression analysis and the data were presented in Table 3. It reveals that good correlation existed between the peak areas concentration of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate. Repeatability was checked by calculating the relative standard deviation (%RSD) of six determinations by injecting six freshly prepared solutions containing and 9 ppm of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate on the same day. The low %RSD values confirm the good precision of the developed method.

Table 3: Linearity plot of 2-Isopropyl-5-Methylcyclohexyldihydroxyacetate concentration range of 9 – 108 ppm.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Peak area</th>
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<tbody>
<tr>
<td>9</td>
<td>98640</td>
</tr>
<tr>
<td>36</td>
<td>394210</td>
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<tr>
<td>54</td>
<td>592411</td>
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<tr>
<td>72</td>
<td>771234</td>
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<tr>
<td>90</td>
<td>974500</td>
</tr>
<tr>
<td>108</td>
<td>1124002</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.9993</td>
</tr>
<tr>
<td>Slope</td>
<td>10456.39748</td>
</tr>
<tr>
<td>Intercept</td>
<td>16097.72165</td>
</tr>
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</table>
6.8. CONCLUSION

The present development study is based on validation of a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantification of 2-Isopropyl-5-Methylcyclohexylidihydroxyacetate in APIs. It has been established that it is highly sensitive with a limit of detection (LOD) of 3 ppm Trace level ammonium acetate is added to the mobile phase to enhance ionization and detection. Selected sample solvents were assessed for the effect on standard stability with and without presence of API. As a systematic approach, it is very important to utilize the comprehensive chromatographic knowledge gained throughout the lifecycle of the development of a drug candidate based on continuous understanding of the API manufacturing process. The method which is able to quantify them at ppm level is developed and validated. We can conclude that the developed method could be very useful for monitoring of 2-Isopropyl-5-Methylcyclohexylidihydroxyacetate in Lamivudine in its pure and tablet form.
6.9. REFERENCES

1. Lamivudine drug profile: www.drugbank.ca/drugs/DB00709


