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# METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF NEVIRAPINE AND LAMIVUDINE IN HUMAN PLASMA BY LC-MS/MS

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**ABSTRACT:**
The present work deals with a simple and accurate liquid chromatography mass spectrometry method for the simultaneous estimation of Nevirapine and Lamivudine from their combination drug product with carbamazepine as internal standard. The proposed LC-MS/MS method utilizes HyPURITY ADVANCE 4.6x50 mm, 5 microm (make thermo) column, at ambient temperature, optimum mobile phase consists of methanol: 5M ammonium acetate buffer (85; 15v/v) and flow rate monitored at 0.800 ml/min. the linearity of the proposed method was investigated in the range of 25.45ng/ml to 4990.23 mg/ml concentrations for Nevirapine and 15.30 mg/ml to 2999.63 mg/ml concentrations for Lamivudine[1]. The mean recoveries were 93.58% and 91.73% for Nevirapine and Lamivudine, respectively. The proposed method was validated for precision, accuracy, linearity, range, robustness and ruggedness. All validation parameters were within the acceptable range. The developed method was successfully applied to estimate the amount of Nevirapine and Lamivudine in combined dosage forms.

**KEYWORDS:** Nevirapine, Lamivudine, robustness, Chromatography.

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## INTRODUCTION

Method Development is done by LC-MS/MS (liquid chromatography is combined with mass spectrometers). Liquid chromatography is a fundamental separation technique in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for nonvolatile and thermally fragile molecules, liquid chromatography can safely separate a wide range of organic compounds, from small molecule drug metabolites to peptides and proteins[1]. The analytical methods reported for this drug include quantization by Nevirapine and Lamivudine by a reversed-phase HPLC and HPTLC method for determination of the drug in human plasma. Lamivudine is a nucleoside reverse transcriptase inhibitor[2]. It is a synthetic nucleoside analogue with activity against HIV-1 and HBV. Lamivudine is a non nucleoside reverse transcriptase inhibitor (NRTIS). It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis.

## MATERIALS AND METHODS

### Materials

Nevirapine and Lamivudine were pure drugs collected from the Hetero Pharmaceuticals Pvt. Ltd. as a gift sample. All other chemicals and reagents were analytical grade purchased from Merck ltd[4].

### METHODS

**1. Protein Precipitation Method:**

In protein precipitation, biological samples (e.g. plasma) are diluted with a protein precipitating

reagent, such as Acetonitrile, typically at a volume ratio between 1:3 and 1:4. The diluted sample is vortexed, and the resulting precipitated proteins are removed using filtration or centrifugation methods. The filtrate or supernatant is analyzed without further processing by LC-MS or LC-MS-MS systems[5,6,7].

**2. Liquid-liquid Extraction Method:**

Liquid-liquid extraction (LLE) is another classic sample preparation technique in which an immiscible solvent is added to the biological sample and shaken and/or vortexed. After the immiscible layers are allowed to separate, the organic layer is often concentrated or evaporated and reconstituted prior to chromatographic analysis[8,9,10].

**3. Solid Phase Extraction Method[11,12,13,14]:**

Solid-phase extraction (SPE) is a separation process that is used to remove solid or semi-solid compounds from a mixture of impurities based on their physical and chemical properties.

The following section describes the steps involved in a complete solid-phase extraction procedure:

1. Conditioning of the cartridge
2. Equilibration
3. Loading the sample
4. Washing
5. Elution of the fractions

**TRIAL: 1**

**Mobile Phase (10mM Ammonium acetate: Methanol, 30:70%, v/v)**

300 mL of HPLC grade methanol was transferred to a 1000 mL reagent bottle and 700 mL of 10mm ammonium acetate buffer was added to it. It was mixed well, sonicated in an ultrasonic bath for 2 to 5 minutes[15, 16].

**Diluent: (Water: Methanol, 50:50) v/v**

A mixture of HPLC methanol and Milli Q water was prepared in the volume ratio of 50:50 as diluent and used also Rinsing Solution. It was then sonicated in an ultrasonicator for 2 to 5 minutes[17].

**Chromatographic Conditions**

**Flow rate :** 0.8ml/min
Column : HyPURITY ADVANCE, 4.6 x 50 mm, 5µm (Make: Thermo)
Run Time : 3.0 min
Injection volume : 15 µL
Observation: The peak shape of Nevirapine and Lamivudine is not good.

**TRIAL: 2**

**Chromatographic Conditions[18]:**

Flow rate : 1.0 ml/min
Column : HyPURITY ADVANCE, 4.6 x 50 mm, 5 µm
Run time : 3.0 min
Injection volume: 15 µL
Observation**:** The retention time was too long and tailing was observed

**TRIAL: 3**

**Chromatographic Conditions:**

Flow rate : 0.8 ml/min
Column : HY-PURITY ADVANCE, 4.6 x 50 mm, 5 µm (Make: Thermo)
Rétention time : Nevirapine 1.00 ± 0.3 minutes, Lamivudine 1.00 ± 0.3 minutes,
Carbamazepine 1.00 ± 0.3 minutes
Injection volume : 15 µL
Run time : 2.5 mins
Observation: The peak shape of Nevirapine and Lamivudine is good and also optimum resolution was obtained with optimum retention time.
Therefore conditions of **trial-3** were optimized.

**Optimized Method for Assay:**

A summary of the chromatographic conditions is as follows:

Column : HY-PURITY ADVANCE, 4.6 x 50 mm, 5 µm
Mobile phase : Methanol: 5 mM Ammonium Acetate buffer (85:15v/v)
Rinsing solution : Water: Methanol (40:60)
Flow rate : 0.800 mL / minute
Detection : Positive ion mode (API 4000)
Nevirapine m/z : - 267.30 (parent) and 226.10 (product)
Lamivudine m/z : – 230.00 (parent) and 112.10 (product)
Carbamazepine m/z:– 237.20 (parent) and 194.10 (product)
Retention time : Nevirapine- 1.00±0.3 minutes,
Lamivudine- 1.00±0.3 minutes,
Carbamazepine- 1.00±0.3 minutes
Sample Cooler
Temperature : 5°C
Injection volume : 15 µL
Rinsing volume : 500 µL (before and after aspiration)
Split ratio : 50:50
Total Run time : 2.50 minutes

## RESULTS AND DISCUSSION

### 1. CHROMATOGRAPHY

Representative chromatograms of aqueous standard analyte and internal standard mixture, blank plasma, blank plasma with internal standard, LLOQ QC sample, LQC sample, MQC sample and HQC sample of Nevirapine, Lamuvidine, and Carbamazepine are given in Figures 1 to 6.

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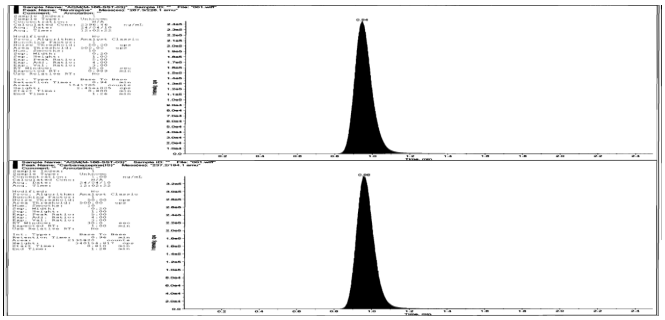

**Fig. No. 1** Representative Chromatogram of an aqueous standard and Internal standard Mixture of Nevirapine

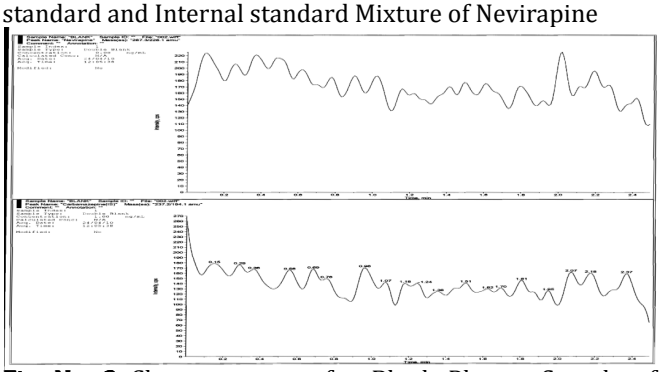

**Fig. No. 2** Chromatogram of a Blank Plasma Sample of Nevirapine

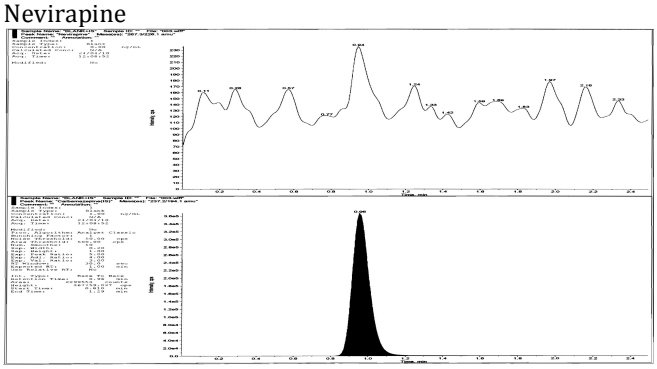

**Fig. No. 3** Representative Chromatogram of Blank Plasma with Internal Standard Sample of Nevirapine

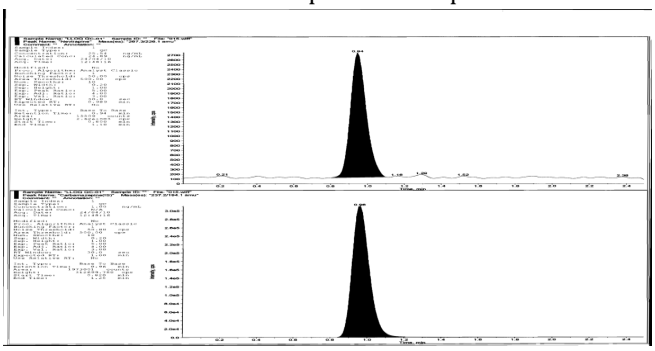

**Fig. No. 4** Representative Chromatogram of a LLOQ QC Sample of Nevirapine

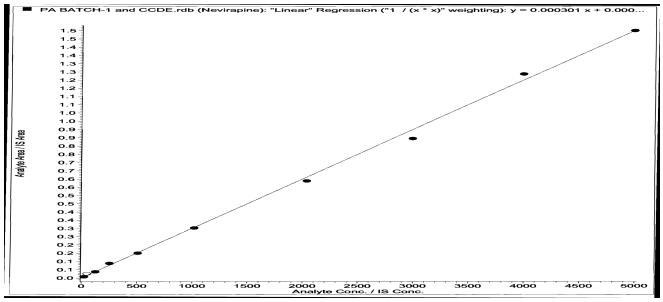


**Fig. No. 5** Representative Calibration Curve for Regression Analysis of Nevirapine

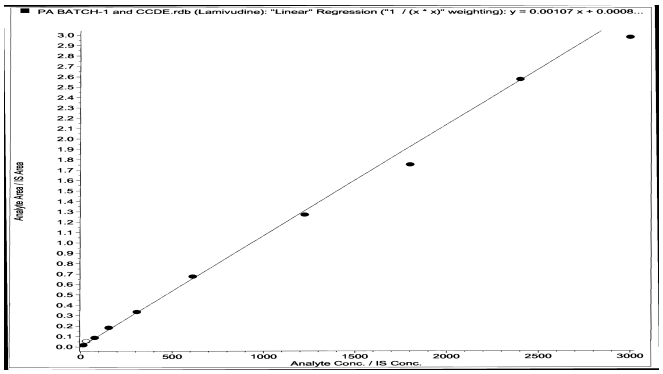


**Fig. No. 6** A Representative Calibration Curve for Regression Analysis of Lamivudine

## 2. SELECTIVITY

Representative chromatogram of extracted blank plasma sample is given in fig. no 2.

### Observation

No significant interference from endogenous components was observed at the mass transitions of Nevirapine, Lamuvidine and internal standard in all the batches, which were screened.

## 3. SENSITIVITY

The lowest limit of reliable quantification for Nevirapine and Lamivudine was set at the concentration of the LLOQ 25.58 and 15.35 mg/mL, the precision and accuracy for Nevirapine, Lamivudine at this concentration was found to be 3.22%, 96.80%, and 2.05% , 89.65% respectively. No statistical outlier was found.

## 4. MATRIX EFFECT

No significant matrix effect was observed in all the eight batches of plasma for Nevirapine and Lamuvidine at low (LQC) and high (HQC) concentrations. The precision and accuracy for Nevirapine, Lamuvidine metabolite at LQC concentration was found to be 2.20% and 93.11% and 3.30% and 99.77% respectively and HQC concentration was found to be 0.69%, 93.49% and 0.53%, 92.97% respectively.

**5. LINEARITY**

Correlation coefficient ($r^2$) was greater than 0.99 in the concentration range of 25.45 mg/mL to 4990.23 mg/mL for Nevirapine. Correlation co-efficient ($r^2$) was greater than 0.99 on the concentration range 15.30 mg/mL to 2999.63 mg/mL for Lamuvidine

**6. PRECISION AND ACCURACY**

The precision of the assay was measured by the percent coefficient of variation over the concentrations of LLOQ QC, LQC, MQC and HQC samples respectively during the course of validation.

**Within-Batch Precision and Accuracy**

**Nevirapine:** Within- ranged from 3.06% to 6.73% (LLOQ QC), 0.71% to 7.20%(LQC, MQC1, MQC2 & HQC) and the within batch accuracy ranged from 90.26% to 95.37%(LLOQ QC), 89.66% to 99.52% (LQC, MQC1, MQC2 & HQC) without statistical outlier.

**Lamivudine:** Within-batch precision ranged from 2.03 % to 2.85 %( LLOQ QC), 0.95% to 9.17% (LQC, MQC1, MQC2 & HQC) and the within batch accuracy ranged from 88.99% to 101.87% (LLOQ QC), 90.53% to 109.74% (LQC, MQC1, MQC2 & HQC) without statistical outlier.

**Ruggedness**

One precision and Accuracy batch (P&A-V) was performed with different column of same make and with different analyst. Within batch precision and accuracy of Nevirapine was ranged from 0.71% to 7.20% and 90.72 % to 99.52% respectively, refer table 3a. Within batch precision and accuracy of Lamuvidine was ranged from 0.95% to 9.17% and 89.99 % to 109.74% respectively, refer table 3.

**7. STABILITIES**

**STANDARD STOCK SOLUTION STABILITY**

Room Temperature Stock Solution Stability

Room temperature stock solution stability was carried out at 07 hours for Nevirapine and Lamuvidine metabolite by injecting six replicates of prepared stock dilutions of Nevirapine equivalent to the middle concentration.

**Room Temperature Spiking Solution Stability**

Comparison of the mean area response at 7 hours was carried out against the Zero hour samples for Nevirapine and Carbamazepine and 6 hours for Lamivudine. The room temperature spiking solution stability of Nevirapine, and Carbamazepine at 7 hours was found to be 99.67%, and 103.49%, respectively. And precision of Nevirapine and Carbamazepine at 7 hours was found to be 3.45%, and 8.86%, respectively.

**Refrigerated Stock Solution Stability: (at 2-8°C)**

Refrigerated stock solution stability of Nevirapine, Lamuvidine, and Carbamazepine was carried out by injecting six replicates of its stock dilutions. The sixth day stock solution stability of Nevirapine, Lamuvidine and Carbamazepine was found to be 99.44%, 103.02% and 102.27% respectively.

**Plasma samples Stability at -20°C**

Nevirapine percent nominal ranged from 96.07% to 102.05% and the precision ranged from 1.46% to 1.36%. No statistical outlier was found. Lamuvidine percent nominal range from 107.85% to 103.77% and the precision ranged from 1.61% to 1.31%. No statistical outlier was found.

**Freeze-thaw Stability**

Six replicates of LQC and HQC were analysed after six freeze-thaw cycles. The percent nominal ranged from 98.49% to 105.78% and 105.84% to 106.02% for six freeze-thaw cycles and the precision ranged from 4.88% to 1.19% and 1.82% to 0.89%. No statistical outlier was found.

**Short-term Room Temperature Stability**

Nevirapine, Lamivudine were found to be stable up to 9.0 hours. The percent nominal ranged from 97.48% to 102.28% for 9.0 hours. The precision ranged from 2.62% to 1.19% for 9.0 hours. No statistical outlier was found.

**Bench top stability**

Nevirapine, Lamivudine were found to be stable up to 9 hours. The stability ranged from 97.48% to 102.28% for 9 hours. The precision ranged from 1.19% to 2.62% for 9 hours. No statistical outlier was found.

**Recovery**

The mean overall recovery of Nevirapine, Lamivudine and Carbamazepine were 93.58%, 91.73% and 99.63% respectively with a precision of 4.34%, 4.37% and 3.98%. No statistical outlier was found.

**Reinjection Stability**

Nevirapine percent nominal at 30 hours ranged from 92.11% to 89.84% and precision ranged from 2.65% to 1.07% and no statistical outlier was found for 0 and 30 hours. Lamivudine percent nominal at 30 hours ranged from 104.10% to 89.54% and precision ranged from 1.39% to 1.01% and no statistical outlier was found for 0 and 30 hours.

**8. DILUTION INTEGRITY**

Nevirapine and Lamivudine twelve sets of dilution integrity samples were prepared by spiking 1.6 times highest standard concentration (7984.37 and 4799.41 ng/mL). Six sets of dilution integrity samples were processed by diluting them twice and another six sets by diluting them four times. Lamivudine precision and accuracy for dilution factor of 2 was 8.44% and 104.74%.

**Table No. 1** Concentration-response Linearity Data for Sensitivity and Matrix effect of Nevirapine

| Nevirapine | Concentration (mg/mL) | | | | | | | | | | Slope | Intercept | R | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | STD-A | STD-B | STD-C | STD-D | STD-E | STD-F | STD-G | STD-H | STD-I | STD-J | | | | |
| CC# | 25.58 | 51.16 | 127.91 | 255.82 | 511.65 | 1023.30 | 2046.59 | 3009.69 | 4012.93 | 5016.16 | | | | |
| 1 | 25.41 | 51.33 | 131.42 | 255.86 | 507.51 | 1010.81 | 2072.73 | 2957.79 | 4027.40 | 4999.68 | 0.0003 | 0.0000 | 0.999 | **0.9998** |
| % Nominal | **99.34** | **100.33** | **102.74** | **100.02** | **99.19** | **98.78** | **101.28** | **98.28** | **100.36** | 99.67 | | | | |

**Table No. 2** Concentration-response Linearity Data for Sensitivity and Matrix effect of Lamivudine

| Lamivudine | Concentration (mg/mL) | | | | | | | | | | Slope | Intercept | R | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | STD-A | STD-B | STD-C | STD-D | STD-E | STD-F | STD-G | STD-H | STD-I | STD-J | | | | |
| CC# | 15.35 | 30.71 | 76.77 | 153.55 | 307.10 | 614.19 | 1228.38 | 1806.44 | 2408.59 | 3010.74 | | | | |
| 1 | 14.99 | 31.40 | 79.14 | 161.85 | 312.45 | 623.38 | 1226.72 | 1778.36 | 2301.11 | 2845.19 | 0.0010 | 0.0008 | 0.9993 | **0.9986** |
| % Nominal | **97.65** | **102.25** | **103.09** | **105.41** | **101.74** | **101.50** | **99.86** | **98.45** | **95.54** | **94.50** | | | | |

**Table No. 3** Matrix Effect of Nevirapine

| Plasma | Nevirapine-ME QC# | Concentration (mg/mL) | | | Mean | Nevirapine-ME QC# | Concentration (mg/mL) | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|
| | | LQC | | | | | HQC | | | |
| | | 68.70 | | | | | 4163.41 | | | |
| 1 | 1,2,3 | 63.92 | 62.56 | 65.72 | 64.07 | 1,2,3 | 3888.50 | 3933.88 | 3876.12 | 3899.50 |
| 2 | 1,2,3 | 62.68 | 63.70 | 63.55 | 63.31 | 1,2,3 | 3877.65 | 3828.64 | 3828.72 | 3845.00 |
| 3 | 1,2,3 | 60.49 | 62.43 | 63.13 | 62.02 | 1,2,3 | 3870.32 | 3885.54 | 3939.27 | 3898.38 |
| 4 | 1,2,3 | 67.93 | 63.91 | 66.52 | 66.12 | 1,2,3 | 3852.82 | 3963.77 | 3953.20 | 3923.26 |
| 5 | 1,2,3 | 64.13 | 62.50 | 63.66 | 63.43 | 1,2,3 | 3896.30 | 3861.03 | 3888.32 | 3881.88 |
| 6 | 1,2,3 | 65.19 | 66.06 | 63.26 | 64.84 | 1,2,3 | 3950.12 | 3846.77 | 3919.12 | 3905.34 |
| Mean | | | | | 63.963 | | | | | 3892.227 |
| S.D. | | | | | 1.4089 | | | | | 26.6942 |
| C.V.(%) | | | | | 2.20 | | | | | 0.69 |
| % Nominal | | | | | 93.11 | | | | | 93.49 |
| N | | | | | 6 | | | | | 6 |

**Table No. 4** Matrix Effect of Lamivudine

| Plasma | Lamivudine-ME QC# | Concentration (mg/mL) | | | Mean | Lamivudine ME QC# | Concentration (mg/mL) | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|
| | | LQC | | | | | HQC | | | |
| | | 41.40 | | | | | 2508.95 | | | |
| 1 | 1,2,3 | 41.82 | 41.85 | 41.98 | 41.88 | 1,2,3 | 2323.39 | 2308.76 | 2361.95 | 2331.37 |
| 2 | 1,2,3 | 41.05 | 41.13 | 39.93 | 40.70 | 1,2,3 | 2357.79 | 2339.43 | 2319.88 | 2339.03 |
| 3 | 1,2,3 | 40.40 | 40.46 | 39.25 | 40.04 | 1,2,3 | 2350.23 | 2331.90 | 2342.03 | 2341.39 |
| 4 | 1,2,3 | 46.17 | 39.01 | 46.20 | 43.79 | 1,2,3 | 2297.11 | 2320.45 | 2306.82 | 2308.13 |
| 5 | 1,2,3 | 40.65 | 39.61 | 41.24 | 40.50 | 1,2,3 | 2316.34 | 2327.40 | 2372.70 | 2338.81 |
| 6 | 1,2,3 | 40.51 | 40.74 | 41.46 | 40.90 | 1,2,3 | 2390.03 | 2308.17 | 2312.47 | 2336.89 |
| Mean | | | | | 41.303 | | | | | 2332.603 |
| S.D. | | | | | 1.3643 | | | | | 12.4585 |
| C.V.(%) | | | | | 3.30 | | | | | 0.53 |
| % Nominal | | | | | 99.77 | | | | | 92.97 |
| N | | | | | 6 | | | | | 6 |

**Table No. 5:** Within Batch Precision and Accuracy for Nevirapine

| Nevirapine | LLOQ QC | LQC | MQC1 | MQC2 | HQC |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| **QC#** | 25.54 | 68.48 | 547.84 | 2490.20 | 4150.34 |
| 1 | 24.89 | 64.49 | 518.47 | 2293.11 | 3694.53 |
| 2 | 22.44 | 63.91 | 515.51 | 2324.44 | 4095.30 |
| 3 | 24.73 | 66.66 | 528.78 | 2253.13 | 4102.65 |
| 4 | 23.33 | 63.96 | 526.24 | 2301.43 | 4224.30 |
| 5 | 22.59 | 63.26 | 512.35 | 2281.13 | 4061.93 |
| 6 | 23.16 | 64.31 | 527.90 | 2322.98 | 4025.37 |
| **Mean** | 23.523 | 64.432 | 521.542 | 2296.037 | 4034.013 |
| **S.D.** | 1.0524 | 1.1706 | 7.0028 | 26.9575 | 179.3204 |
| **C.V.%** | 4.47 | 1.82 | 1.34 | 1.17 | 4.45 |
| **% Nominal** | 92.10 | 94.09 | 95.20 | 92.20 | 97.20 |
| **N** | 6 | 6 | 6 | 6 | 6 |
| 7 | 24.53 | 66.85 | 539.82 | 2357.68 | 3929.96 |
| 8 | 23.97 | 67.04 | 575.66 | 2315.29 | 4083.68 |
| 9 | 23.77 | 66.58 | 575.20 | 2324.56 | 3976.46 |
| 10 | 23.31 | 66.06 | 500.75 | 2298.02 | 3925.43 |
| 11 | 22.19 | 65.42 | 505.21 | 2277.47 | 3946.02 |
| 12 | 22.20 | 63.51 | 489.66 | 2336.50 | 3961.80 |
| **Mean** | 23.328 | 65.910 | 531.050 | 2318.253 | 3970.558 |
| **S.D.** | 0.9613 | 1.3139 | 38.2495 | 28.3148 | 58.6429 |
| **C.V.%** | 4.12 | 1.99 | 7.20 | 1.22 | 1.48 |
| **% Nominal** | 91.34 | 96.25 | 96.94 | 93.10 | 95.67 |
| **N** | 6 | 6 | 6 | 6 | 6 |

## SUMMARY AND CONCLUSION

Pharmaceutical analysis simply means analysis of pharmaceuticals. Today pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. The pharmaceutical industry is under increased scrutiny from the government and the public interested groups to contain costs and at consistently deliver to market safe , efficacious product that fulfill unmet medical needs. From the optical characteristics of the proposed method, it was found that the drug obeys linearity within the concentration range of 25.45 mg/mL to 4990.23 mg/mL. From the results shown in precision table, indicates that the proposed method has good reproducibility. From the results shown in accuracy and precision tables, indicates that the method is accurate

**Nevirapine**

- Intra-day Precision for Nevirapine ranged from 2.43% to 5.02% and the accuracy ranged from 91.38% to 96.37%
- Between batch precision ranged from 2.88% to 4.96% and the between batch accuracy ranged from 92.11% to 96.45%. No statistical outlier was found.
- Within-batch precision ranged from 0.71% to 7.20% and the within batch accuracy ranged from 89.66% to 99.52% without statistical outlier.

**Lamivudine:**

- Between batch precision ranged from 3.62% to 5.61% and the between batch accuracy ranged from 95.57% to 105.76%. No statistical outlier was found.
- Within-batch precision ranged from 0.95% to 9.17% and the within batch accuracy ranged from 89.99% to 109.74% without statistical outlier.
- Intra-day Precision ranged from 2.16% to 6.68% and the accuracy ranged from 93.79% to 104.44%. No statistical outlier was found. Thus the purpose of the present investigation was success.

In conclusion, the results indicate that the proposed method was precise, accurate, linear and able to quantify the Nevirapine and Lamivudine.

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