International Journal of Pharmacy and Natural Medicines

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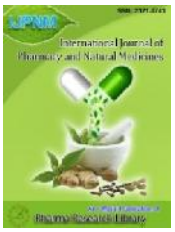

Research Article Open Access

# Analytical Method Development and Validation for the Simultaneous Estimation of Lamivudine and Raltegravir by RP-HPLC Method

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**A B S T R A C T**

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Lamivudine and Raltegravir in tablet dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. From literature review and solubility analysis initial chromatographic conditions Mobile phase ortho phosphoric acid buffer: Acetonitrile 40:60 were set (Buffer $P^H$ 2.45 adjusted with Triethylamine), Symmetry C18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 260 nm. As the methanol content was increased Lamivudine and Raltegravir got eluted with good peak symmetric properties. The retention times for Lamivudine and Raltegravir was found to be 2.335 min and 3.400 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to150 % levels, $R^2$ value was found to be as 0.999. By using above method assay of marketed formulation was carried out, 100.7% was present. Full length method was not performed; if it is done this method can be used for routine analysis of Lamivudine and Raltegravir.

**Keywords:** HPLC, Ortho Phosphoric Acid, Acetonitrile, Symmetry C18, Lamivudine, Raltegravir

**A R T I C L E I N F O**

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**Article History: Received** 15 April 2017, **Accepted** 25 May 2017, **Available Online** 15 June 2017

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Manuscript ID: IJPNM3491

**PAPER-QR CODE**

**Citation:** B. Priyanka, *et al.* Analytical Method Development and Validation for the Simultaneous Estimation of Lamivudine and Raltegravir by RP-HPLC Method. *Int. J. Pharm. Natural Med*., 2017, 5(1): 43-49.

## 1. Introduction

Analytical chemistry is a branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative analysis or detection of compounds and quantitative analysis of the compounds. A qualitative method yields information about the identity of atomic or molecular species or functional groups in the sample. A quantitative method, in contrast provides numerical information as to the relative amount of one or more of these components.

**Classification of Analytical Methods**

Analytical methods are classified into two categories; they are classical methods and instrumental methods.

**Classical methods**

Analysis of substances was carried out by separating the components of interest in a sample by precipitation, extraction or distillation. For qualitative analysis, the separated components were then treated with reagents that yielded products that could be recognized by their colors, their boiling or melting points, their solubilities in a series of solvents, their optical activities, their odors or their refractive indexes. For quantitative analysis, the amount of analyte was determined by gravimetric or by volumetric measurements. In gravimetric measurements, the mass of the analyte or some compound produced from the analyte was determined. In volumetric measurements, also called titrimetric analysis, the volume or mass of a standard reagent required to react completely with the analyte is measured.

**Instrumental methods**

Measurement of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, fluorescence, mass-to-ratio began to be used for quantitative analysis of various inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative and quantitative determination. These newer methods for separating and determining chemical species are collectively known as instrumental methods of analysis. Most of the instrumental methods fit into one of the following three categories viz, spectroscopy, electrochemistry and chromatography.

**Advantages of instrumental methods:**

- Small samples can be used
- High sensitivity is obtained
- Measurements are reliable
- Determination is very fast
- Complex samples can be analyzed

**Limitations of instrumental methods:**

- An initial or continuous calibration is required
- Sensitivity and accuracy depend on the instrument
- cost of equipment is large
- concentration range is limited
- specialized training is needed

**Chromatography:** Chromatography is relatively a new technique which was first invented by M.Tswett, a botanist in 1906. Chromatography was derived from Greek words chroma and graphos meaning “colour” and “writing” respectively. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation. Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. Chromatography may be preparative or analytical. The purpose of preparative Chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical Chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analytes in a mixture.

The chromatographic method of separation, in general, involves the following steps:

1. Adsorption or retention of substance on the stationary phase.
2. Separation of adsorbed substances by mobile phase.
3. Recovery of the separated substances by continuous flow of the mobile phase; the method being called elution.
4. Qualitative and quantitative analysis of the eluted substances.

**High Performance Liquid Chromatography (HPLC)**

A variety of methods are available for analyzing pharmaceutical compounds. High Performance/Pressure Liquid Chromatography (HPLC) is one of the best methods of choice for analyzing a variety of natural and synthetic compounds. It is because it offers high performance over ambient pressure.[3] The phenomenal growth in chromatography is largely due to the introduction of the technique called high-pressure liquid chromatography, which is frequently called high-performance liquid chromatography (both are abbreviated as HPLC). It allows separations of a large variety of compounds by offering some major improvements over the classical column chromatography, TLC, GC; and it presents some significant advantages over more recent techniques such as supercritical fluid chromatography (SFC), capillary electrophoresis (CE), and electro kinetic chromatography. [4] Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization. Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture components. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture.

Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of Chromatographic methods.

The four main types of HPLC techniques are

- Normal-Phase Chromatography.
- Reversed-Phase Chromatography.
- Ion-Exchange Chromatography.
- Size-Exclusion Chromatography.

**Normal-Phase Chromatography**

The term "normal phase" is used to denote a chromatographic system in which a polar stationary phase is employed and a less polar or non-polar mobile phase is used for elution of the analytes. In the normal-phase mode, neutral solutes in solution are separated on the basis of their polarity; the more polar the solute, the greater is its retention on the column. Since the mobile phase is less polar than the stationary phase, by increasing the polarity of the mobile phase results in decreased solute retention. Normal-Phase chromatography is most commonly applied to the analysis of samples that are soluble in non-polar solvents and it is particularly well suited to the separation of isomers and to class separations. Although the separation mode has occasionally been misidentified as reversed phase, it is normal phase by virtue of the fact that increased aqueous levels of the mobile phase reduce carbohydrate retention, and elution order follows carbohydrate polarity. Normal-phase separations have occasionally been combined off-line with Reversed-phase chromatography to separate a wider range of species than could be accomplished by either technique alone. The feasibility of such a system, however, is contingent on the compatibility of the normal-phase eluent with that of the reversed-phase column.

**Reversed-Phase Chromatography**

Reversed-Phase Chromatography, the most widely used chromatographic mode, is used to separate neutral molecules in solution on the basis of their hydrophobicity. As the name suggests, Reversed-Phase Chromatography is the reverse of Normal-Phase Chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, a decrease in the polarity of the mobile phase results in a decrease in solute retention. Modern Reversed-Phase Chromatography typically refers to the use of chemically bonded stationary phases, where a functional group is bonded to silica, for this reason, Reversed-Phase Chromatography is often referred to in the literature as Bonded-Phase Chromatography. Occasionally, however, polymeric stationary phases such as polymethacrylate or polystyrene, or solid stationary phases such as porous graphitic carbon, are used. Weak acids and weak bases, for which ionization can be suppressed, may be separated on reversed-phase columns by the technique known as ion suppression. In this technique a buffer of appropriate pH is added to the mobile phase to render the analyte neutral or only partially charged. Acidic buffers such as acetic acid are used for the separation of weak acids, and alkaline buffers are used for the separation of weak bases.

The analysis of strong acids or strong bases using reversed-phase columns is typically accomplished by the technique known as ion-pair chromatography (also commonly called paired-ion or ion-interaction chromatography). In this technique, the pH of the eluent is adjusted in order to encourage ionization of the sample; for acids pH 7.5 is used, and for bases pH 3.5 is common.

Reversed-Phase Chromatography is the most popular mode for the separation of low molecular weight (<3000), neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids, vitamins, and β-blockers. Because of the mobile phase in Reversed-Phase Chromatography is polar, Reversed-Phase Chromatography is suited to the separation of polar molecules that either are insoluble in organic solvents or bind too strongly to the polar, normal-phase materials.

## 2. Materials and method

**Materials**

Tri ethyl amine, $KH_2PO_4$, Water and Methanol for HPLC, Acetonitrile for HPLC, Ortho phosphoric Acid

**Methodology**

**HPLC Method Development**

**Selection of chromatographic condition**

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

**Selection of detection wavelength:**

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected. Standard solutions of Lamivudine and Raltegravir were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 260 nm was selected as the detection wavelength for the present study.

**System suitability:**

A Standard solution of Lamivudine and Raltegravir working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

## 3. Results and Discussion

### Method Validation

**Linearity**

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

**Specificity**:

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc

**Precision**:

The precision of the method was demonstrated by intra-day and inter-day precision studies. Intra-day studies were performed by injecting three (3) repeated injections within a day. Peak area and %RSD were calculated and reported

**Intermediate Precision:**

Intermediate precision of the analytical method was determined by performing method precision on another day by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and mean %assay value, standard deviation & %RSD was calculated.

**Accuracy**:

- Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.
- Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.
- Percentage method: For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.
- Acceptance criteria: The mean % recovery of the Lamivudine and Raltegravir at each level should be not less than 95.0% and not more than 105.0%.

**Limit of Detection and Limit of Quantification:**

The Sensitivity of measurement of Lamivudine and Raltegravir by use of the proposed method was estimated in terms of the Limit of Detection (LOD) and the Limit of Quantitation (LOQ). The LOD and LOQ were calculated by the use of the equations:

**Robustness:**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of a method's robustness, deliberate change in the Flow rate was made to evaluate the impact on the method.

**System suitability:**

A Standard solution of Lamivudine and Raltegravir working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from five replicate injections.

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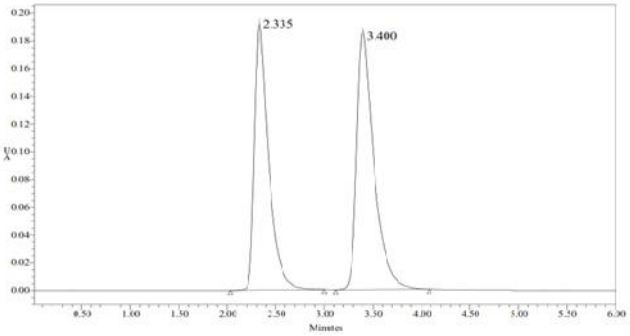


**Figure 1:** Chromatogram of Trail-5

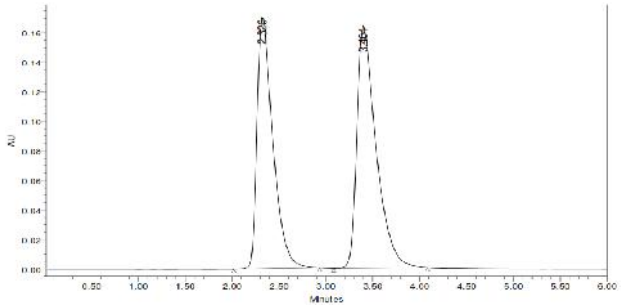


**Figure 2:** Chromatogram of sample system suitability

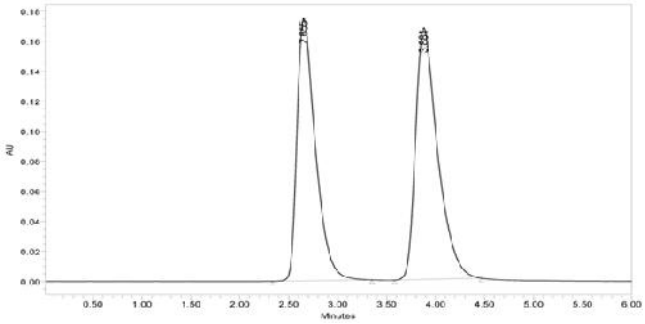


**Figure 3:** Representative Chromatogram at Flow rate of 0.8 ml/min

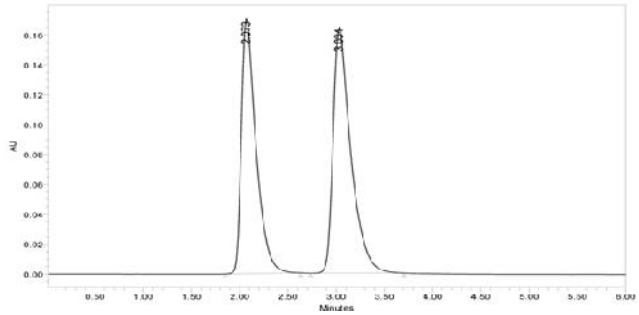


**Figure 4:** Representative Chromatogram at Flow rate of 1.2 ml/min

**Table 1:** Chromatographic Conditions

| **Parameters** | **Description** |
|---|---|
| Flow rate | 1ml $min^{-1}$ |
| Column | Symmetry $C_{18}$ Column (250mm x 4.6mm)5µm. |
| Mobile Phase | ACN: Phosphate buffer $P^H$ 2.5 (60:40 v/v) |
| Buffer | Potassium dihydrogen orthophosphate PH 2.5 adjusted with Orthophosphoric acid |
| Detector | PDA |
| Column temperature | Ambient |
| Type of elution | Isocratic |
| Wavelength | 260 nm |
| Injection volume | 20µl |
| Run time | 10min |

**Table 2:** Chromatographic conditions of trail 5

| **Parameters** | **Description** |
|---|---|
| Flow rate | 1ml $min^{-1}$ |
| Column | Symmetry $C_{18}$ Column (250mm x 4.6mm)5µm. |
| Mobile Phase | Phosphate buffer: acetonitrile $P^H$ 2.5 (40:60 v/v) |
| Buffer | Potassium dihydrogen orthophosphate PH 2.5 adjusted with Orthophosphoric acid |
| Detector | PDA |
| Column temperature | Ambient |
| Type of elution | Isocratic |
| Wavelength | 260nm |
| Injection volume | 20µl |
| Run time | 10min |

**Table 3:** Preparation of working standard solutions for Linearity

| **Sample ID** | **Lamivudine** | | **Raltegravir** | |
|---|---|---|---|---|
| | **Concentration (mcg/ml)** | **Area** | **Concentration (mcg/ml)** | **Area** |
| 20% of operating concentration | 20 | 226418 | 10 | 277182 |
| 40% of operating concentration | 30 | 432920 | 15 | 521695 |
| 60% of operating concentration | **40*** | **677256** | **20*** | **808274** |
| 80% of operating concentration | 50 | 869825 | 25 | 1033875 |
| 100% of operating concentration | 60 | 1095759 | 30 | 1285804 |
| Correlation Coefficient | | | 0.999 | |

**Table 4:** Accuracy Study of Lamivudine

| **Sample Id** | **Conc found (µg/ml)** | **Conc. Obtained (µg/ml)** | **%Recovery** | **Mean recovery** | **Statistical Analysis** |
|---|---|---|---|---|---|
| **50%** | 5 | 5.01 | 100.2 | | **%RSD= 0.505** |
| **50%** | 5 | 4.96 | 99.2 | 99.73 | |
| **50%** | 5 | 4.99 | 99.8 | | |
| **100%** | 10 | 9.95 | 99.5 | | **%RSD=0.66** |
| **100%** | 10 | 9.87 | 98.7 | 98.8 | |
| **100%** | 10 | 9.82 | 98.2 | | |
| **150%** | 15 | 14.64 | 97.6 | | **%RSD=1.45** |
| **150%** | 15 | 14.76 | 98.4 | 98.8 | |
| **150%** | 15 | 15.06 | 100.4 | | |

**Table 5:** Accuracy Study of Raltegravir

| **Conc (µg/ml)** | **Concn Obtained(µg/ml)** | **%Recovery of drug** | **Mean accuracy** | **%RSD** |
|---|---|---|---|---|
| 5 | 4.92 | 98.0 | | |

| | | | | |
|---|---|---|---|---|
| 5 | 4.96 | 99.2 | | |
| 5 | 5.02 | 100.4 | **99.2** | **1.2** |
| 10 | 9.95 | 99.5 | | |
| 10 | 9.94 | 99.4 | | |
| 10 | 9.98 | 99.8 | **99.5** | **0.2** |
| 15 | 14.78 | 98.6 | | |
| 15 | 14.94 | 99.6 | **99.0** | **0.530** |
| 15 | 14.83 | 98.8 | | |

**Table 6:** Precision Results for Lamivudine

**Name: Lamivudine**

| | Name | RT | Area |
|---|---|---|---|
| 1 | Lamivudine | 2.335 | 1963566 |
| 2 | Lamivudine | 2.332 | 1964716 |
| 3 | Lamivudine | 2.333 | 1965030 |
| 4 | Lamivudine | 2.330 | 1960856 |
| 5 | Lamivudine | 2.331 | 1966445 |
| Mean | | | 1964123 |
| Std. Dev. | | | 2094.9 |
| % RSD | | | 0.11 |

**Table 7:** Precision Results for Raltegravir

**Name: Raltegravir**

| | Name | RT | Area |
|---|---|---|---|
| 1 | Raltegravir | 3.408 | 2304558 |
| 2 | Raltegravir | 3.406 | 2299453 |
| 3 | Raltegravir | 3.409 | 2296908 |
| 4 | Raltegravir | 3.404 | 2295001 |
| 5 | Raltegravir | 3.407 | 2299613 |
| Mean | | | 2299107 |
| Std. Dev. | | | 3597.7 |
| % RSD | | | 0.16 |

**Table 8:** Intermediate Precision Results for Lamivudine

**Name: Lamivudine**

| | Name | RT | Area |
|---|---|---|---|
| 1 | Lamivudine | 2.332 | 1984822 |
| 2 | Lamivudine | 2.331 | 1985152 |
| 3 | Lamivudine | 2.330 | 1985353 |
| 4 | Lamivudine | 2.332 | 1987338 |
| 5 | Lamivudine | 2.330 | 2004113 |
| Mean | | | 1989356 |
| Std. Dev. | | | 8308.1 |
| % RSD | | | 0.42 |

**Table 9:** Intermediate Precision Results for and Raltegravir

**Name: raltigravir**

| | Name | RT | Area |
|---|---|---|---|
| 1 | raltigravi | 3.413 | 2316744 |
| 2 | raltigrav | 3.409 | 2314478 |
| 3 | raltigravi | 3.408 | 2314400 |
| 4 | raltigravi | 3.412 | 2313639 |
| 5 | raltigrav | 3.408 | 2332909 |
| Mean | | | 2318434 |
| Std. Dev. | | | 8174.5 |
| % RSD | | | 0.35 |

## 4. Conclusion

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Lamivudine and Raltegravir in tablet dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. From literature review and solubility analysis initial chromatographic conditions Mobile phase ortho phosphoric acid buffer:Acetonitrile 40:60 were set (Buffer $P^H$ 2.45 adjusted with Triethylamine), Symmetry C 18 (250×4.6mm, 5µ) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 260 nm. As the methanol content was increased Lamivudine and Raltegravir got eluted with good peak symmetric properties. The retention times for Lamivudine and Raltegravir was found to be 2.335 min and 3.400 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to150 % levels, $R^2$ value was found to be as 0.999.By using above method assay of marketed formulation was carried out, 100.7% was present.Full length method was not performed; if it is done this method can be used for routine analysis of Lamivudine and Raltegravir.

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