INTRODUCTION

High Performance Liquid Chromatography is one of the widely used analytical techniques used for the separation, identification and quantification of components in a mixture. This technique is mostly suitable for non-volatile compounds, thermally unstable compounds and compounds having high molecular weights. HPLC Depends on pumps to pass the pressurized liquid solvent containing the sample mixture through a column filled with a solid material. The components present in the sample interact differently with the adsorbent material, causing different flow rates for the different components of the mixture which leads to the separation of the components.

Basic Principles of HPLC: HPLC is similar to liquid chromatographic technique, where separation between the mobile phase (solvent) and a stationary phase (column packing) depending upon the nature of the stationary phase.

1) Adsorption chromatography, where the separation is mainly based on the repeated adsorption-desorption steps.

2) Partition chromatography where the separation is based on partition between the mobile and the stationary phase.

3) Ion-exchange chromatography where the stationary phase is made up of ionic surface of opposite charge to that of the sample.

4) Size exclusion chromatography where the sample is separated according to its molecular size through a column filled with a material having small controlled pore size.

Based on Modes of Chromatography there exist two modes of chromatography depending upon the polarity (polar or non-polar) of the stationary and the mobile phase used.

Normal Phase: NP-HPLC is a type of partition chromatographic technique where stationary phase is polar and the mobile phase is non-polar.

Reverse Phase HPLC: RP-HPLC is the most commonly used HPLC technique for the analysis of wide range of pharmaceutical compounds. In this, the stationary phase used is non-polar while the mobile phase is polar in nature.

Based on Elution Technique There are two types of HPLC techniques based on elution.

Isocratic Separation

Gradient Separation

Isocratic Elution: Mobile phase composition remains constant throughout the experimental procedure is known as isocratic elution. Isocratic elution increases the retention time of peak and leads to the disadvantage that late-eluting peaks giving very flat and broad. Best for simple separations of samples.

Gradient Elution: A separation in which the mobile phase composition is changed during the separation process is known as a gradient elution. Gradient elution decreases...
the retention time of the later-eluting components so that they elute faster, giving narrower peaks. Which also improves the peak shape and the peak height. Best for the separation of complex samples and also used in method development for unknown mixtures.

Based on The Scale of Operation: Analytical HPLC: This technique is employed only for the analysis of the sample. Since, the quantity of the sample being used is very low, its recovery for re-utilization is not feasible.

Preparative HPLC: In this technique, after the analysis, individual fractions of the pure compounds are recovered using fractional collector and re-utilized⁶.

**Instrumentation of HPLC:**

HPLC Instrument Consists of The Following:

1. **Mobile phase Reservoirs**
2. **Solvent delivery pumps**
3. **Pumps**
4. **Sample injection systems.**
5. **Columns**
6. **Detectors.**
7. **Recorders and integrators.**

**Mobile Phase Reservoirs:** Mobile phase reservoir which will carry the mobile phase solution and through the pump the solution in the reservoirs will be pumped into the column. The mobile phase reservoir contains different polarity of solvents such as water, methanol, and acetonitrile. The purity of solvents and inorganic salts used to make the mobile phase is paramount⁷. A general rule of thumb is to use the highest purity of solvent that is available and practical depending on the particular application. The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

Pumps: Pump refers to the device that forces the mobile phase through a liquid chromatography column at pressures up to 10,000 psi. There are 3 Types of HPLC Pumps:

- **Syringe pump**
- **Constant Pressure Pumps**
- **Reciprocating pump or Constant flow pump**

**Syringe Type HPLC Pump:** It is also known as Displacement pumps. They are large stainless-steel motor driven hypodermic needles. They act by maintaining a constant flow of the mobile phase throughout the column and it is not affected by any changes. Disadvantages: It has a limited solvent capacity (250-500ml) and it also requires refilling of solvent chamber for continual use.

**Pneumatic or Constant Pressure Pumps:** These Pumps give a non-pulsating solvent flow but this pump has very small capacity. Constant pressure pumps also known as non- reciprocating pumps. In these pumps, the mobile phase is placed in a collapsible container and highly pressurized gas is passed into the pump.

**Reciprocating Pumps:** This pump consists of a small chamber in which the mobile phase is pumped by both forward and backward movements of piston which runs on an electric motor. The flow of the mobile phase in the chamber is regulated by two valves. There are two types of reciprocating pumps:

1. **Single Acting Piston Pump**
2. **Dual acting piston pump**

Advantages:

1. Small solvent chamber volume (35-400 Gl)
2. High output pressure (up to 10,000 psi)
3. Constant flow rate
4. Independent of column back pressure and solvent viscosity.

**Injector:**

Stop Flow: In which the flow of mobile phase is stopped for a while and the sample is injected through a valve device.

**Rheodyne Injector:** It is the most popular injector. This has a fixed volume loop like 20µL or 50µL or more. Injector has 2 modes, i.e., load position when the sample is loaded in the loop and inject mode when the sample is injected.

**Columns:** The column considered the “heart of the chromatograph” the separation of the sample components is achieved when those components pass through the column. The column’s stationary phase separates the sample components of interest using various physical and chemical parameters. Column material: - The column is made up of stainless steel, glass, poly-ethylene and PEEK (poly ether ether ketone). Most widely used as a stainless steel this can withstand high pressure.

**Guard column:** this type of columns will have very small quantity of absorbent it improves the life of the analytical column. They also act as pre-filter to remove particulate matter, if any, and other material. Analytical column: - The most important part of the HPLC technique which decides the efficiency of separation. These are several stationary phases available depending upon the technique or mode of separation used.

Columns Different types of columns on the basis of their composition and method of separation:

1. Normal phase columns
2. Reverse phase columns
3. Ion exchange columns
4. Size exclusion columns

**Detectors:** Detector is a device used to detect components in the mixture being eluted off the chromatographic column. The detectors convert a change in effluents into...
an electric signal that is recorded by data system. There are many different types of detectors that can be used for HPLC. The detector is used to sense the presence of a compound passing through and to provide an electronic signal to a data acquisition device. The main types of detectors used in HPLC are refractive index (RI), ultraviolet (UV-Vis) and fluorescence, but there is also diode array, electrochemical and conductivity detectors. Each detector has its assets, limitations and sample types for which it is most effective. Most applications in drug analysis use detectors that respond to the absorption of ultraviolet radiation (or visible light) by the solute as it passes through the flow-cell inside the detector. The recent development of the so-called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include liquid chromatography mass spectrometry (LC-MS), liquid chromatography-mass spectrometry-mass spectrometry (LC-MSMS), liquid chromatography-infrared spectroscopy (LC-IR) and liquid chromatography-nuclear magnetic resonance (LCNMR). These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR [10]. Types of Detectors:

1. Absorbance (UV with Filters, UV with Monochromators)
2. IR Absorbance
3. Fluorescence
4. Refractive-Index
5. Evaporative Light Scattering Detector (ELSD)
6. Electrochemical
7. Mass-Spectrometric
8. Photo-Diode Array

Recorders and Integrators: They record the signals emerging from the detector as deviations from the baseline. The electrical signals obtained from the detector are amplified (if necessary) and recorded as a function of time with the help of a potentiometric recorder. The responses are obtained in the form of chromatographic peaks from which the retention time of the solute molecules is determined. They possess data processing ability and record the individual peaks with their retention time, height and width, peak area, percentage of area, etc. Therefore, they help to overcome the disadvantage associated with detectors.

Experimental methodology: The drug was weighed using CONTECH analytical weighing balance and the glassware used was borosilicate glass. The solutions were accurately transferred using micropipette Fact A (Sr no.388516). Before the introduction of the mobile phase into the HPLC system it has been sonicated using LABOTECH Sonicator (LMUC-2, L8486) and filtered using vacuum filtration and adjusted pH is checked with ELICO pH meter. For the quantitative estimation of Valacyclovir HCl by chromatographic method, HPLC system of make SHIMADZU which has a binary pump (LC 20AD) and the column employed was PHENOMENEX C18 and the sample was injected using Rheodyne injector and it was detected using UV detector and the system connected to it has installed software called LC SOLUTIONS for data interpretation.

MATERIALS AND METHODS

Valacyclovir Hydrochloride pure drug was obtained as a gift sample from Gland Pharma Hyderabad, India. Valacyclovir HCl formulation (VALCIVIR) was purchased from the local drug store. HPLC grade Methanol and HPLC grade Water and Glacial Acetic acid from SD-Fine Chemicals, Mumbai, India. Hydrogen Peroxide (H2O2), Hydrochloric acid (HCl), and sodium hydroxide (NaOH) used were of Analytical grade.

Method Development Using Reverse Phase-High Performance Liquid Chromatography.

Based on drug solubility and P Ka value following conditions have been used to develop the method estimation of Valacyclovir HCl

Method Development Trials

Preparation of Standard Solution: Accurately weighed 10mg of the drug and transferred into 10ml of Volumetric Flask the volume was made up to the mark using diluent as HPLC grade water. To get the concentration of 1000µg/ml.

Trial 1: Chromatographic conditions:

System: Shimadzu LC-20AD
Column: PHENOMENEX C18, 250mmx4.6mmx5µ
Mobile Phase: Methanol: Water [30:70]
Flow rate: 0.8ml/min
Detection: UV at 251nm
Temperature: 25°C
Injection Volume: 20µl
Run time: 10minutes
Pump Mode: Isocratic

Figure 1: Chromatogram of Valacyclovir HCl with HPLC grade Methanol: Water (30:70)
Table 1: Chromatogram of Valacyclovir HCl with Methanol: Water (30:70)

| Peak# | Ret. time | Area  | T. Plate# | Tailing F. |
|-------|-----------|-------|-----------|------------|
| 1     | 1.608     | 13130 | 2378.747  | 1.251      |

Inference: In this trial Peak splitting and other peaks were observed so the further trial was carried out.

**Preparation of Valacyclovir Hydrochloride Solutions**

Preparation of Standard Stock Solution: Accurately weighed 10mg of the drug and transferred into 10ml of Volumetric Flask the volume was made up to the mark using diluent as HPLC grade water. To get the concentration of 1000µg/ml. From this 1ml was pipetted out and transferred into a 10ml volumetric flask and diluted with water to obtain a concentration of 100µg/ml (secondary stock solution).

Preparation of Working Standard Solution: From the standard stock solution 0.25ml, 0.75ml,1ml ,1.25ml, and 1.50ml of aliquots were transferred into a series of 10ml Volumetric Flask and diluted with HPLC grade water to get the concentrations of 25,50,100,125, and 150µg/ml.

Preparation of Valacyclovir Hydrochloride Sample Solution: 5 tablets of Valacyclovir HCl formulation were weighed and powdered. Powdered weight equivalent to 10mg was transferred into 10ml Volumetric Flask. The drug was dissolved using diluent as HPLC grade Water and the solution was subjected to sonication for 10min the solution was filtered and the volume was made up to the mark using diluent. To obtain the concentration of 1000µg/ml.

**Method Validation**

The developed method was validated as per ICH guidelines Q2 (R1).

**Specificity:** Checking the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific. From the standard stock solution (1000µg/ml), 1 ml was pipetted out and transferred into a 10ml volumetric flask and the volume was made up to the mark with diluent.

**System Suitability:** The standard solutions were injected five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits from the standard stock solution (100µg/ml), 1 ml was pipetted out and transferred into 10ml volumetric flask and the volume was made up to the mark with diluent.

**Linearity:** Linearity was performed by injecting Valacyclovir HCl standard stock solution in the range of 25 to 150 µg/ml and the response was recorded and calibration curve was plotted by taking concentrations on X-axis and peak areas on Y-axis and R2 value was determined by plotting the calibration curve.

**Precision:** The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from the multiple sampling of the same homogeneous sample under the prescribed conditions. (Intraday and inter day precision). Precision was assessed by injecting Valacyclovir HCl 100 µg/ml concentration of 6 replicate injections into HPLC system. % RSD was calculated

**Accuracy:** The accuracy of the proposed method was assessed by recovery studies. Valacyclovir HCl standard solution was spiked to sample solution at three concentration levels (50%, 100%, and 150%). three replicates of each concentration level were prepared and injected into HPLC system. And response was recorded. % Recovery was calculated

Preparation of 50µg/ml Sample Stock Solution (50µg/ml Solution): From the sample stock solution (1000 µg/ml), 0.5ml was pipetted and transferred into 10ml volumetric flask and the volume were made up to mark with diluent to obtain 50µg/ml concentration

Preparation of 25µg/ml from Standard Stock Solution: From the standard stock solution (1000µg/ml), 0.25ml was pipetted and transferred into 10ml volumetric flask and the volume was made up to the mark with diluent.

Preparation of 50µg/ml from Standard Stock Solution: From the standard stock solution (1000µg/ml), 0.5ml was pipetted and transferred into 10ml volumetric flask and the volume was made up to the mark with diluent.

Preparation of 75µg/ml Standard Stock Solution: From the standard stock solution (1000µg/ml), 0.75ml was pipetted and transferred into another 10ml volumetric flask and the volume was made up to the mark with diluent.

Preparation of 50% Solution: Pipette 2ml of 25µg/ml standard solution was spiked to 2ml of 50µg/ml sample solution of Valacyclovir Hydrochloride and the solution was mixed and injected into HPLC System.

Preparation of 100% Solution: Pipette 2ml of 50µg/ml standard solution was spiked to 2ml of 50µg/ml sample solution of Valacyclovir Hydrochloride and the solution was mixed and injected into HPLC System.

Preparation of 150% Solution: Pipette 2ml of 75µg/ml standard solution was spiked to 2ml of 50µg/ml sample solution of Valacyclovir Hydrochloride and the solution was mixed and injected into HPLC System.

**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. Robustness method was determined by injecting three replicates of 100µg/ml Valacyclovir HCl solution by varying the flow rate in chromatographic condition at 0.8±0.1ml/min. Chromatograms were obtained and %RSD was calculated.
L.O.D & L.O.Q: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. Whereas the L.O.Q is the lowest amount of sample which can be quantitatively determined with suitable precision and accuracy.

Assay of Valacyclovir Hydrochloride

Preparation of Sample Stock Solution (1000µg/ml Solution): 5 tablets were weighed and calculated average weight of each tablet. Then powered it, the tablet powder was taken equivalent to 10mg in 10ml volumetric flask. Then add 3/4th of the diluent and sonicated for 10min, filtered it and made up with diluent. (1000 µg/ml of Valacyclovir Hydrochloride).

Preparation of Sample Working Solutions (100µg/ml Solution): From the above filtered sample stock, 1ml of solution was pipette out and transferred to 10ml volumetric flask and the volume was made up to the mark with diluent. (100µg/ml of Valacyclovir Hydrochloride) Inject 20µl of 100 µg/ml sample and standard 100 µg/ml solutions into HPLC System and % recovery was calculated using formula:

\[
\text{% assay} = \left( \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{weight of standard}}{\text{dilution of standard}} \times \frac{\text{dilution of sample}}{\text{purity}} \times \frac{\text{weight of sample}}{\text{weight of tablet}} \times \frac{\text{lab claim}}{100} \right) \times 100
\]

RESULTS AND DISCUSSION

Chromatographic Method: The goal of this work is to develop a sensitive and precise method for quantification of Valacyclovir Hydrochloride

Validation Parameters

Specificity

No peaks were observed at the RT of Valacyclovir Hydrochloride when blank was injected.
Table 3: Linearity Data.

| Concentration | Area  | Ret. time | T. plate count | Tailing factor |
|---------------|-------|-----------|----------------|----------------|
| 100ppm        | 4621204 | 2.242   | 2942.399       | 1.048          |

**Accuracy**

Three Concentrations of Valacyclovir HCl at 50%, 100%, 150% spiked to sample were injected in a triplicate manner.

**Assay**

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using before mentioned formula.

\[
\% \text{ assay} = \left( \frac{\text{sample area}}{\text{weight of standard}} \times \frac{\text{dilution of sample area}}{\text{weight of sample}} \times \frac{\text{purity}}{100} \right) \times 100
\]

Percentage Assay = \left( \frac{4599889}{4621204} \times 10 \times 10 \times \frac{98.5}{100} \times \frac{770}{500} \times 100 \right)

Percentage Assay = 0.980 x 100

Percentage Assay = 98.0%

**CONCLUSION**

Proposed RP-HPLC method was found to be simple, specific, precise, accurate, rapid and economical for the estimation of Valacyclovir Hydrochloride in bulk and Pharmaceutical dosage form. The developed method was validated statistically according to ICH guidelines. The sample recovery in formulation was in good agreement with their respective label claims.

For the proposed method, mobile phase used was HPLC grade Methanol: water (60:40) pH 3.5 with glacial acetic acid PHENOMENEX C18 Column (250x4.6mm) fpm, flow rate 0.8ml/min, eluents were scanned with UV detector in system at 251nm. The retention time for Valacyclovir Hydrochloride was found to be 2.24mins

This method was found to be better than previously reported methods. Hence above method can be used in quality control for routine analysis of tablets of Valacyclovir Hydrochloride.

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