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RESEARCH ARTICLE

Analytical Method Development and Validation of Remdesivir in Bulk and Pharmaceutical Dosage Forms Using Reverse-Phase-High Performance Liquid Chromatography

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ABSTRACT

Background: Remdesivir has received significant attention for its potential application in the treatment of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Remdesivir has already been tested for Ebola virus disease treatment and found to have activity against SARS and MERS coronaviruses. The remdesivir core contains GS-441524, which interferes with RNA-dependent RNA polymerases alone. In non-human primates, following IV administration, remdesivir is rapidly distributed into PBMCs and converted within 2 h to the active nucleoside triphosphate form, while GS-441524 is detectable in plasma for up to 24 h. Nevertheless, remdesivir pharmacokinetics and pharmacodynamics in humans are still unexplored, highlighting the need for a precise analytical method for remdesivir and GS-441524 quantification. **Objectives:** The validation of a reliable UHPLC-MS/MS method for remdesivir and GS-441524 quantification in human plasma. **Methods:** Remdesivir and GS-441524 standards and quality controls were prepared in plasma from healthy donors. Sample preparation consisted of protein precipitation, followed by dilution and injection into the QSight 220 UHPLC-MS/MS system. Chromatographic separation was obtained through an Acquity HSS T3 1.8 µm, 2.1 × 50 mm column, with a gradient of water and acetonitrile with 0.05% formic acid. The method was validated using EMA and FDA guidelines. **Results:** Analyte stability has been evaluated and described in detail. The method successfully fulfilled the validation process and it was demonstrated that, when possible, sample thermal inactivation could be a good choice to improve biosafety. **Conclusions:** This method represents a useful tool for studying remdesivir and GS-441524 clinical pharmacokinetics, particularly during the current COVID-19 outbreak.

Keyword: Remdesivir, Corona Virus, Reversed-phase-high performance liquid chromatography

INTRODUCTION

Drug

A drug may be defined as a substance meant for diagnosis, cure mitigation, prevention or treatment of diseases in human beings or animals or for altering any structure or any function of the body[1].

Drugs play a key role in the progress of human civilization by curing diseases. Today majority of the drug used are of synthetic origin. These are produced in the bulk and used for their therapeutic effects in pharmaceutical formulations[2]. There are biologically active chemical substances generally formulated into convenient dosage forms such as tablets, capsules, ointments, and injectables, these formulations deliver the drug substance in stable, on-toxic and acceptable form, ensuring its bioavailability, and therapeutic activity.

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Quality, safety, and efficacy of drugs

Safety and efficacy are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological-toxicological[3] profile as well as the adverse effects caused by the impurities in bulk and dosage forms. The impurities in drugs often possess unwanted pharmacological or toxicological effect by which any benefit from their administration may be outweighed.

Every country has legislation on bulk drugs and their pharmaceutical formulations that set standards and obligatory quality indices for them. These regulations are presented in separate articles, journals, and specific relating to individual drug and published in the form of book called as pharmacopoeias[4].

- Indian Pharmacopoeia –(IP)
- British Pharmacopoeia-(BP)
- United states Pharmacopoeia-USP
- European Pharmacopoeia-EP

Nowadays, drug introduced in to the market have been increasing at a rapid rate. These drugs may be either new entities or partial structural modifications of the existing drugs and which are included in one or few pharmacopoeias only, after time lag because of several reasons. Under these conditions, standards and analytical procedures for these drugs may not be inaccessible. Hence, it becomes necessary to develop newer analytical for such drugs.

The quality and safety of the drugs are generally assured by monitoring and controlling the release of drug and level of impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis.

Introduction of pharmaceutical analysis

Pharmaceutical analysis simply means analysis of pharmaceuticals. Webster' dictionary defines a pharmaceutical is a medical drug[5]. A more appropriate term for a pharmaceutical is active pharmaceutical ingredient (API) or active ingredient[6] to distinguish it from a formulated product or drug product is prepared by formulating a drug substance with inert ingredient (excipients)[7] to prepare a drug product that is suitable for administration to patients.

Research and development (R & D) play a very comprehensive role in new drug development and follow-up activities to ensure that a new drug product meets the established standards is stable and continue to approved by regulatory authorities, assuring that all batches of drug product are made to the specific standards utilization of approved ingredients and production method becomes the responsibility of pharmaceutical analysts in the quality control (QC)[8] or quality assurance[9] department. The methods are generally developed in an analytical R & D department and transferred to QC or other departments as needed. At times they are transferred to other divisions.

By now it should be quite apparent that pharmaceutical analysts play a major role in assuring the identity, safety, efficacy, and quality of drug product, safety and efficacy studies required that drug substance and drug product meet two critical requirements.

1. Established identity and purity.
2. Established bio availability/dissolution.

Analytical chemistry

A branch of chemistry deals with the identification of compounds and mixtures (qualitative analysis) or the determination of the proportions of the constituents (quantitative analysis). The techniques commonly used are titration, precipitation, spectroscopy, chromatography, etc.[10].

Analytical chemistry serves the needs of many fields

- In industry analytical chemistry provides the means of testing raw materials[11] and for assuring the quality of finished products whose chemical composition is critical. Many household products, fuels, paints, pharmaceuticals, etc., are analyzed by the procedures developed by analytical chemists before being sold to the consumer.
- The nutritional value of food is determined by chemical analysis for major components such as protein and carbohydrates and trace components such as vitamins and minerals. Indeed even, the calories in a food are often

calculated from its chemical analysis.
- In medicine, analytical chemistry is the basis for clinical laboratory tests, which help physicians to diagnose disease and chart the progress in recovery.
- Environmental quality is often evaluated by testing for suspected contaminants using the techniques of analytical chemistry.
- Analytical chemists also make important contributions to fields as diverse as forensic chemistry, archaeology, and space science.

Chromatography

Chromatography is a family of analytical chemistry techniques for the separation of mixtures[12]. It involves passing the sample, a mixture that contains the analyte, in the "mobile phase," often in a stream of solvent, through the "stationary phase." The stationary phase retards the passage of the components of the sample. When components pass through the system at different rates they become separated in time, like runners in a marathon. Ideally, each component has a characteristic time of passage through the system. This is called its "retention time."

A physical separation method in which the components of a mixture are separated by differences in their distribution between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves through it in a definite direction. The substances must interact with the stationary phase to be retained and separated by it. A chromatograph takes a chemical mixture carried by liquid or gas and separates it into its component parts as a result of differential distributions of the solutes as they flow around or over a stationary liquid or solid phase. Various techniques for the separation of complex mixtures rely on the differential affinities of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass; such as paper, gelatin, or magnesium silicate gel. Analytical chromatography is used to determine the identity and concentration of molecules in a mixture. Preparative chromatography is used to purify larger quantities of a molecular species.

Types of chromatography

- Based on modes of chromatography
  - Normal-phase (NP) chromatography
  - Reverse phase (RP) chromatography
- Based on principles of separation
  - Adsorption chromatography
  - Ion exchange chromatography
  - Ion pair chromatography
  - Size exclusion chromatography
  - Affinity chromatography
  - Chiral phase chromatography
- Based on elution technique
  - Isocratic separation
  - Gradient separation
- Based on the scale of operation
  - Analytical high performance liquid chromatography (HPLC)
  - Preparative HPLC.

Based on modes of chromatography

NP-HPLC

NP liquid-liquid chromatography uses a polar stationary phase and less polar mobile phase[13]. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptane's. If the sample is strongly retained, the polarity of the mobile phase should be increased, perhaps by adding small amounts of methanol or dioxin.

In the NP mode, separations of oil-soluble vitamins, essential oils, nitro phenols, or more polar homologous series have been performed using alcohol/heptane as the mobile phase. Column used in NP chromatography for chiral separation: Chiracel OJ and Chiracel OD.

RP-HPLC

RP chromatography uses hydrophobic bonded packing, usually with an octadecyl or octyl functional group and a polar mobile phase, often a partially or fully aqueous mobile phase[14].

Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. In general, the lower the polarity of the mobile phase, the higher is its eluent strength. The elution order of the classes

of compounds in table is reversed (thus the name reverse-phase chromatography).

Pharmaceutical analysis simply means analysis of pharmaceuticals. Webster' dictionary defines a pharmaceutical is a medical drug. A more appropriate term for a pharmaceutical is API or active ingredient to distinguish it from a formulated product or drug product is prepared by formulating a drug substance with inert ingredient (excipients) to prepare a drug product that is suitable for administration to patients. R & D play a very comprehensive role in new drug development and follow-up activities to ensure that a new drug product meets the established standards is stable and continue to approved by regulatory authorities, assuring that all batches of drug product are made to the specific standards utilization of approved ingredients and production method becomes the responsibility of pharmaceutical analysts in the QC or quality assurance department. The methods are generally developed in an analytical R & D department and transferred to QC or other departments as needed. At times they are transferred to other divisions. By now it should be quite apparent that pharmaceutical analysts play a major role in assuring the identity, safety, efficacy, and quality of drug product, safety and efficacy studies required that drug substance and drug product meet two critical requirements.

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Comparison of NP and RP HPLC

Table 1: Comparison of normal phase and reverse phase HPLC

Properties	Normal Phase	Reversed Phase
Polarity of Stationary Phase	High	Low
Polarity of mobile Phase	Low to medium	Low to high
Sample elution order	Least polar First	Most polar First
Retention will increase by	Increasing surface of stationary phase Increasing of n-alkyl chain length of stationary phase. Decreasing polarity of mobile phase Increasing polarity of sample molecules	Increasing surface of stationary phase. Increasing polarity of mobile phase Decreasing polarity of sample molecules

HPLC: High-performance liquid chromatography

Based on principles of separation

Adsorption chromatography

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

Ion exchange chromatography

Iron exchange chromatography uses a stationary phase support that has been derivative to as to permanently bind charged groups to the surface. The mobile phase is typically an aqueous buffer.

This technique is used primarily for the analysis of ions such as strong acids or bases or for separation of large molecules such as nucleic acids, proteins, or large peptides.

Ion-pair chromatography

Ion-pair chromatography is a "hybrid" technique in which charges groups are temporarily bound to the surface of a "reversed-phase type of column packing. This technique is often used for the analysis of small, weak-acid, or weak-base compounds.

Size-exclusion chromatography

- This separates molecules according to their molecular mass. Larger molecules are eluted first and the smallest molecule last.
- This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

Affinity chromatography

- Affinity chromatography operates using immobilized bio chemical's that have a specific affinity to the compound of interest.
- Separation occurs as the mobile phase and sample pass over the stationary phase. The

Table 2: Limits of HPLC parameters

S. no	Parameters	Recommendations
1	Theoretical plates (N)	>2000
2	Tailing factor (T)	2
3	Resolution (Rs)	>2 between peak of interest and the closest eluting potential interference
4	Repeatability	RSD ≤2%
5	Capacity factor (k^1)	>2.0

HPLC: High performance liquid chromatography

sample compound or compounds of interest are retained as the rest of the impurities and mobile phase through.

- Changing the mobile phase conditions then elutes the compounds.

Chiral phase chromatography

In this type of chromatography, optical isomers are separated using chiral stationary phases. Two approaches for chiral separation are available, indirect, which utilizes derivatizing agents, and direct, which uses chiral stationary phases or chiral mobile phase additives.

Based on elution technique

Isocratic elution

- In isocratic elution a sample is injected to a given column and the mobile phase is unchanged through the time required for the sample components to elute from the column.
- The isocratic separation of samples widely varying with k' (partition ratio) values long elution times.
- To adequately handle samples that have both weakly retained and strongly retained substances, the rates of individual band migrations must be changed.

Gradient elution

- Steady changes of the mobile phase composition during the chromatographic run are called gradient elution. The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved.
- Starting with the low content of the organic component in the eluent we allow the least retained components to be separated. Strongly retained components will sit on the adsorbent surface on the top of the column, or will move very slowly.
- When we start to increase an amount of organic component in the eluent (Acetonitrile) then strongly retained components will move faster and faster, because of the steady increase of the competition for the adsorption sites.
- Gradient elution also increases quasi-efficiency of the column. In the isocratic elution, the longer a component is retained, the wider its peak.
- In gradient elution especially with the smooth gradient shape without flat regions, the tail of the peak is always under the influence of the stronger mobile phase when compared to the peak front. Thus, molecules on the tail of the chromatographic zone (peak) will move faster.
- This will tend to compress zone and narrow the resultant peak. The optimum gradient for a particular separation is selected by trial and error.

Based on the scale of operation

Analytical HPLC

In analytical HPLC, quantitative and qualitative determination of a compound is done. It is the most widely used technique. In this method, the sample amount applied to the column is typically in the microgram range or lower quantities. The mass ratio of compound to the stationary phase on the column is <1: 100000. Under these conditions good separations with sharp and symmetrical peaks are achieved. Recovery of samples is not possible as small quantities of the sample are used.

Preparative HPLC

In preparative HPLC, isolation and purification of a product are done. It is an expensive technique, when compared to the traditional purification techniques such as distillation, crystallization, or extraction.

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and pharmaceutical industry as well as in biotechnology and biochemistry. It starts in the microgram range for isolation of enzymes in biotechnology (micro purification). For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry, it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams.

HPLC

HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric materials, and a wide variety of other high-molecular weight poly functional groups.

Basic principle of HPLC

- HPLC is a separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase.
- The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic conditions, each component in a sample has difference distribution equilibrium depending on solubility in the phases and or molecular size.
- As a result the components move at different speeds over the stationary phase and are thereby separated from each other.
- The column is a stainless steel (or resin) tube, which is packed with spherical solid particles.
- Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector located near the column inlet.
- The injected sample enters the column with the mobile phase and the components in the sample migrate through it passing between the stationary and mobile phases.
- Compound move in the column only when it is in the mobile phase. Compounds that tend to be distributed in the mobile phase therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower.
- In this way, each component is separated on the column and sequentially elutes from the outlet. A detector connected to the outlet of the column detects each compound eluting from the column.

HPLC INSTRUMENTATION

The general instrumentation for HPLC incorporates the following components:

1. There is a solvent reservoir for the mobile phase.
2. The mobile phase must be delivered to the column, by some type of pump. To obtain separations either based on short analysis time or under optimum pressure, a wide range of pressure and flow is desirable. The pumping system must be pulse-free or else have pulse damper to avoid generating baseline instability in the detector.
3. Sampling valves or loops are used to inject the sample in the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of the mobile phase to eliminate an unnecessary peak.
4. Ahead of the separation column there may be a guard column or an in-line filter to prevent contamination of the main column by small particulate.
5. To measure column inlet pressure a pressure gauge is inserted in front of the separation column.
6. The separation column contains the packing needed to accomplish the desired HPLC separation. These may be silica's for adsorption chromatography, bonded phases for liquid-liquid chromatography, exchange functional groups bonded to the stationary support for exchange chromatography, gels of specific porosity for exclusion chromatography, or some other unique packing for a particular separation method.

7. A detector with some type of data handling device completes the basic instrumentation.

Schematic diagram for HPLC instrument

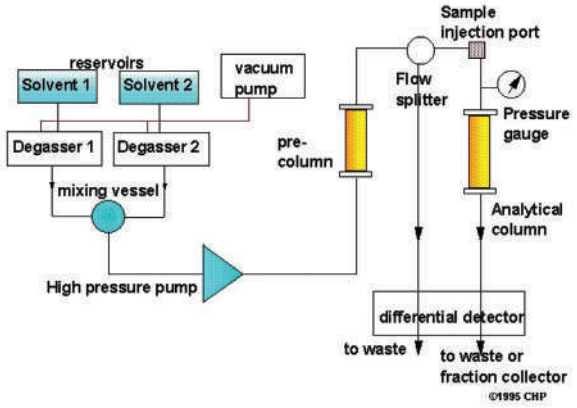


Figure 1: Schematic diagram for high performance liquid chromatography instrument

Mobile -phase delivery system

The mobile phase must be delivered to the column over a wide range of flow rates and pressure. A de gasser is needed to remove dissolved air and other gases from the solvent. Another desirable feature in the solvent-delivery system is the capability for generating a solvent gradient.

Pump

A pump should be able to operate to at least 1500 psi, a pressure suited to less expensive chromatographs. However, 6000 psi is a more desirable pressure limit. For many analytical columns only moderate flow rates of 0.5–2.0 ml/min need to be generated.

Separation columns

Column is the important part of a HPLC instrument Columns are constructed of heavy-wall, glass-lined metal tubing or stainless steel tubing to withstand high pressures (up to 7000 psi) and the chemical action of the mobile phase. Most column lengths range from 10 to 30 cm; short, fast columns are 3 to 8 cm long. For exclusion chromatography, columns are 50 to 100 cm long.

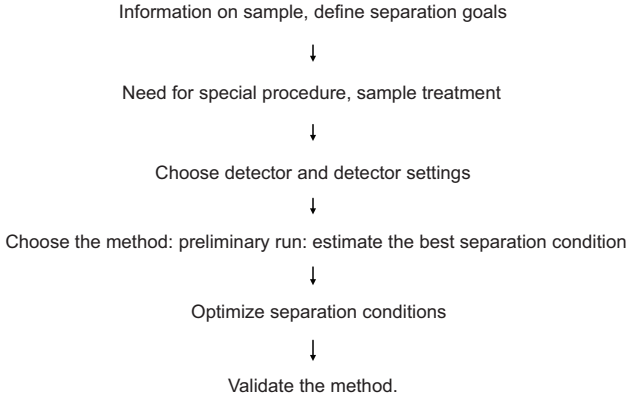


Figure 2: Selection of validation method

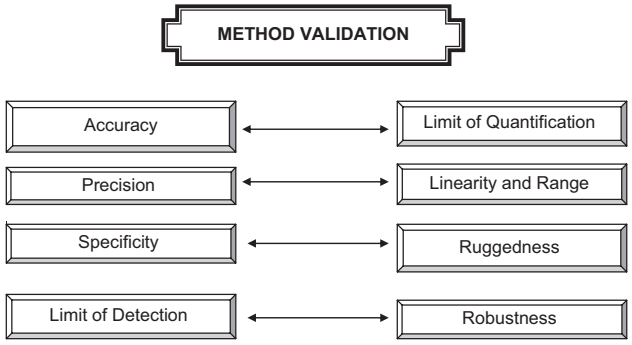


Figure 3: Method validation

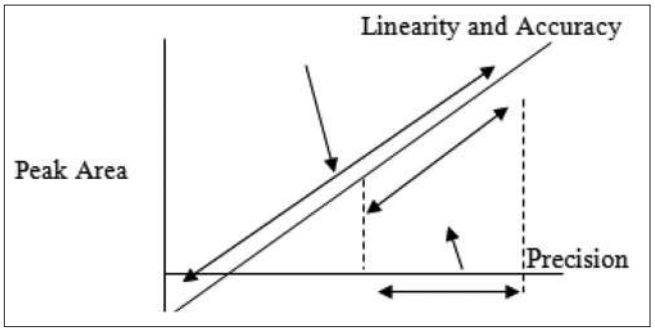


Figure 4: Graph for linearity and accuracy

Standard columns

Many HPLC separations are done on columns with an internal diameter of 4–5 mm. Such columns provide a good compromise between efficiency sample capacity, and the amount of packing and solvent required. Column packing feature particles, that is, uniformly sized and mechanically stable. Particle diameters lie in the range 3–5 µm, occasionally up to 10 µm or higher for preparative chromatography. The columns are classified into various categories depending on their carbon loading.

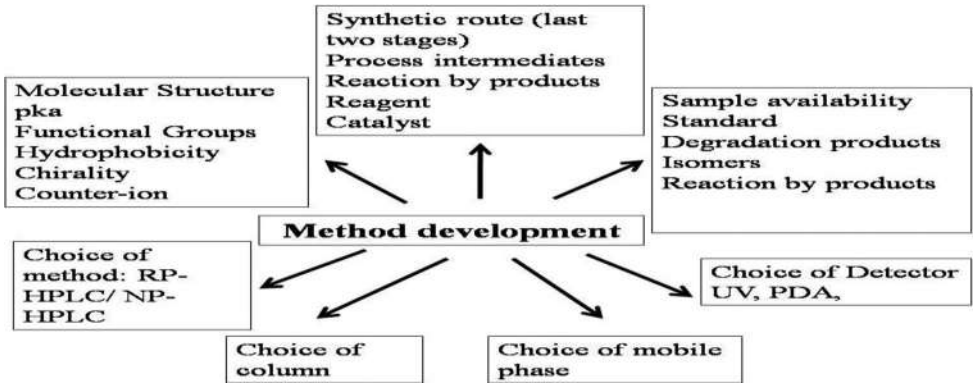


Figure 5: Outline of the process involved in method development

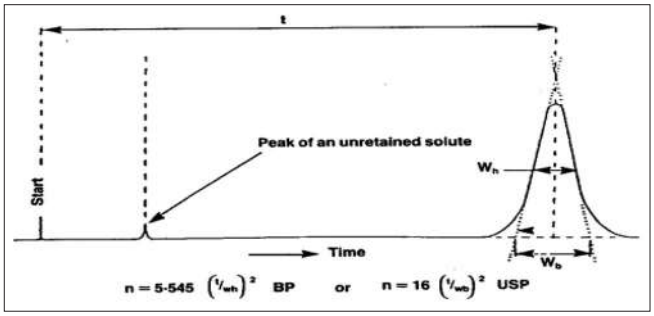


Figure 6: Pictorial representation of number of theoretical plates

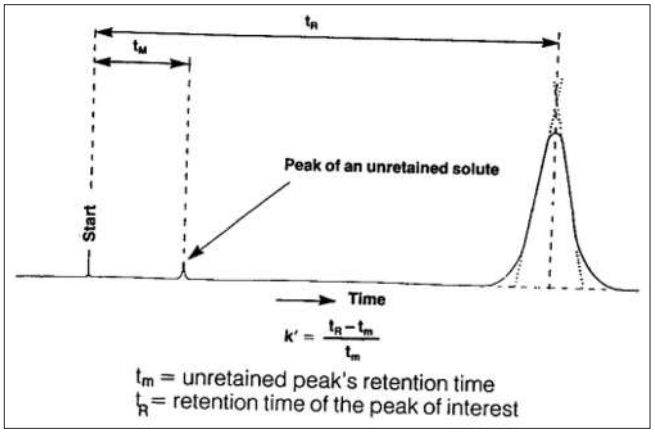


Figure 7: Pictorial representation of capacity factor

Detectors

The sensitivity of universal detector for HPLC has not been devised yet. Thus, it is necessary to select a detector on the basis of the problem.

Ultraviolet (UV) visible photometers and spectrometers

Optical detectors based on UV-visible absorption are the workhorses of HPLC, constituting over 70% of the all detection systems in use. Basically, three types of absorbance detectors are available: A fixed wavelength detector, a variable wavelength detector, and a scanning wavelength[15].

Fixed wavelength detectors

A fixed wavelength detector uses a light source that emits maximum light intensity at one or several discrete wavelengths that are isolated by appropriate filters.

Variable wavelength detector

A variable wavelength detector is a relatively wide-band pass it offers a wide selection of UV and visible wavelength, but at an increased cost.

Photo diode array (PDA) detector

To obtain a real-time spectrum for each solute as it elutes, solid-state diode arrays are required. The diode arrays work in parallel simultaneously monitoring all wavelengths.

Digital electronic integrators are widely used today in HPLC for measuring peak areas. These devices automatically sense peaks and print out the areas in numerical forms. Computing integrators are even more sophisticated and offer a number of features in addition to basic digital integration because these devices have both memory and computing capabilities to upgrade integrating parameters to maintain accuracy as the separation progress and eluting peaks become broader. Many of these

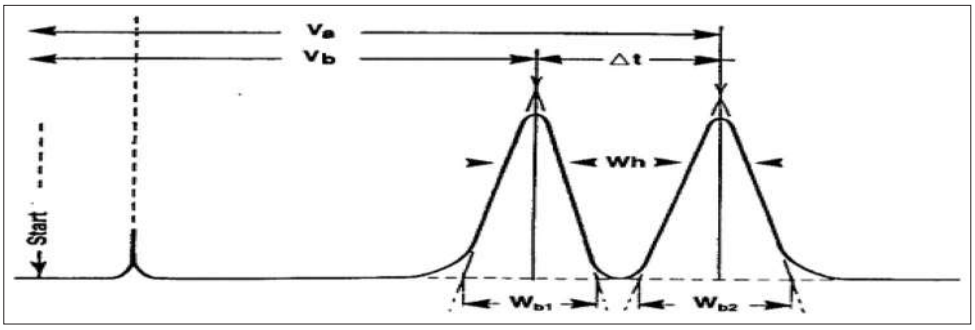


Figure 8: Pictorial representation of resolution

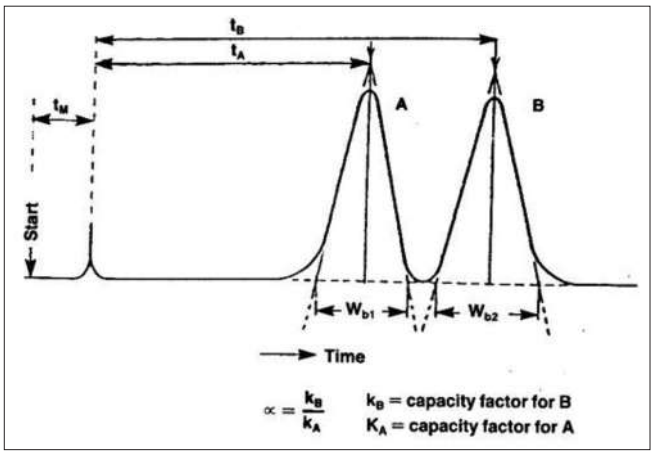


Figure 9: Pictorial representation of selectivity factor

devices print out a complete report including names of the compounds, retention times, peak areas, and area correction factors. With the help of peak area and height values, the peak width can be calculated (considering the peak as a triangle) and it can also be used for the calculation of number of theoretical plates.

Selectivity of HPLC-method development

Most of the drugs can be analyzed by HPLC-method because of several advantages such as rapidity, specificity, accuracy, precision, case of automation, and eliminates tedious extraction and isolation procedures. Some of advantages are:

- Speed (analysis can be accomplished in 20 min or less).
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reliable columns (wide variety of stationary phases)
- Ideal for substances of low volatility
- Easy sample recovery, handling, and maintenance
- Easy programming of the numerous functions in each module.

Time programmable operation sequence, such as initiating operation of detector lamp and pump to obtain a stable base line and equilibrated column before the workday begins.

- Excellent reproducibility of retention times.
- An injection volume variable from 0.1 to 100 µl without any hardware modification.
- The flexibility of data analysis.
- Suitable to avoid any interference from impurity.
- Suitable for preparative liquid chromatography on a much large scale.

Role of the column

The HPLC column is the heart of the method, critical in performing the separation. The column must possess the selectively, efficiency, and reproducibility to provide a good separation. All of these characteristic are dependent on the column manufacture's production of good quality columns and packing materials. Properties of the silica (backbone) such as metal content and silanol activity produced in the manufacturing and bonding process determine the properties of the finished bonded phase. Good silica and bonding process will provide the reproducible and symmetrical peaks necessary for accurate quantitation.

Commonly used reversed phases are C18 (octadecylsilane, USP L1), C8(octylsilane, USP L7), phenyl (USP L11), and cyano (USP L18) [16].

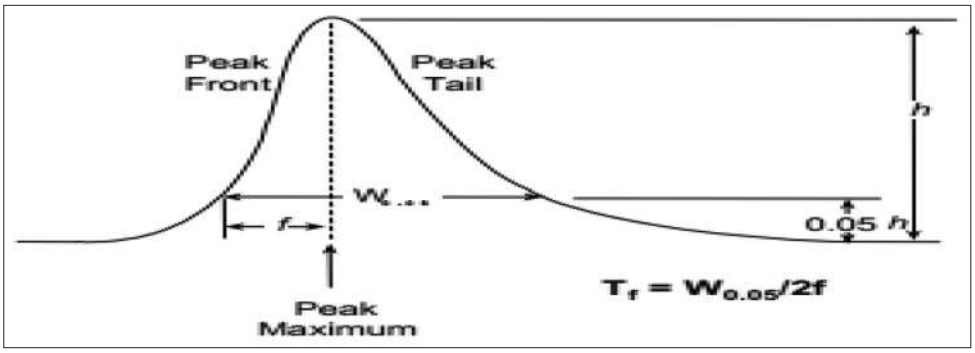


Figure 10: Pictorial representation of peak asymmetry

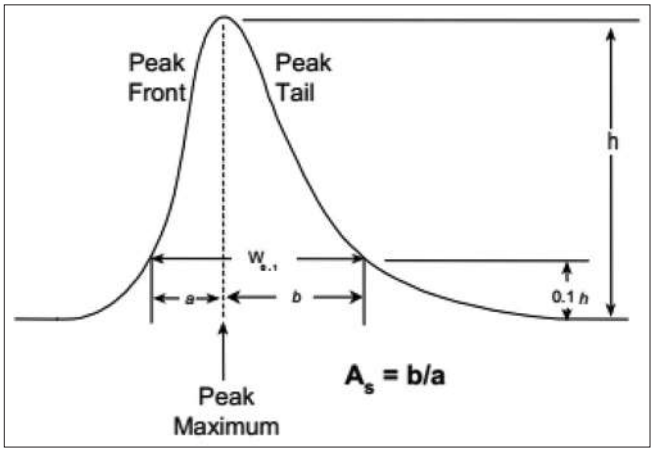


Figure 11: Pictorial representation of peak asymmetry

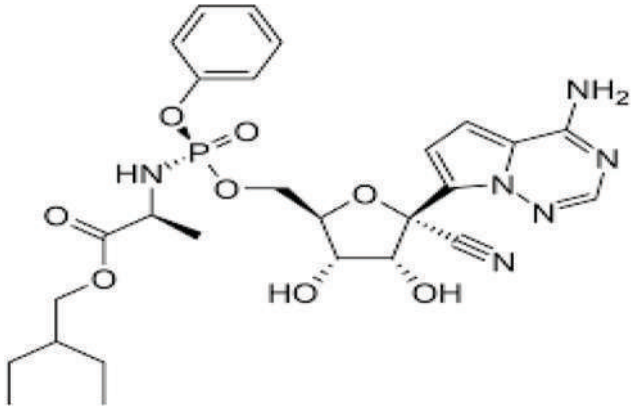


Figure 12: Molecular structure of remdesivir

Initial mobile phase selection

Mobile phase is the most important parameter in RP HPLC. Type of mobile phase used may have a big effect on the retention. It can promote or suppress an ionization of the analyte molecules, and it also can shield an accessible residual silanol or any other active adsorption centers on the adsorbent surface. Proper selection of the mobile phase is the second most important step in the development of the separation method (the first one is the selection of the adsorbent type). The main requirement for the mobile phase is that it has to dissolve the analytes up to the concentration suitable for the detection.

Role of flow rate

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slow flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, to increase the resolution slightly, there is a corresponding increase in the run time.

Role of temperature

While temperature is a variable that can affect selectivity, a, its effect is relatively small. Furthermore, the k ‘ generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Snyder *et al*.[17] reported that an increase of 1°C will decrease the k ‘ by 1–2%, and both ionic and neutral samples.

Role of pH

P^H is another factor in the resolution equation that will affect the selectivity of the separation. In RP HPLC, sample retention increase when the analyte is more hydrophobic. Thus when an acid (HA) or base (B) is ionized (Converted from the unionized free acid or base) it becomes more hydrophilic

(less hydrophobic and more soluble in the aqueous phase) and less interactive with the column's binding sites.

$HA \rightarrow H^+ + A^-$

$B + H^+ \rightarrow BH^+$

Hydrophobic Hydrophilic

(More retained on column) (Less retained on column)

As a result, the ionized analytic is less retained on the column, so that the k ' is reduced, sometimes dramatically. When the pH =pK_a for the analyte, it is half ionized, that is, the concentrations of the ionized and unionized species are equal. As mostly all of the pH-caused changes in retention occur within ±1.5 pH units of the pK_a value, to ensure practically 100% unionization for retention purposes. The pH range most often encountered in RP HPLC is 1–8, normally considered as low pH (i.e., 1–4) and intermediate pH (i.e., 4–8). Generally, at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate range offers an advantage in increased analyte retention and selectivity.

Parameters affecting by the chromatographic conditions

- Column efficiency (N)
- Capacity factor (K')
- Resolution factor (R_S)
- Retention Factor (R_f)
- Retention time (Rt)
- Relative retention (Rr)
- Peak asymmetry factor (As).

Column efficiency (N)

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (N) and can be calculated using the following

$$N = 5.54 \times t_R^2 / W_h^2$$

Where, t_R= Retention time or the baseline distance between the point of injection and the perpendicular dropped from the maximum of the peak of interest. W_h= The width of the peak of interest determined at half peak height, measured in the same units as t_R

The number of theoretical plates can be expressed per meters (N)

$$N = L/H$$

Where, L = length of column in meters; H= height of theoretical plates

The column plate number increases with several factors:

- Well-packed columns (Columns "quality")
- Longer columns
- Lower flow rates (but not too low)
- Smaller column-packing particles
- Lower mobile-phase viscosity and higher temperature
- Smaller sample molecules

Capacity factor (K')

Capacity factor is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula.

$$(K') = t_R - t_M$$

Where,

t_R= retention time of the solute

t_M= retention time of an unretained component.

A low Dm value indicates that the peak elutes close to the solvent front, which may compromise selectivity. A minimum Dm value of 1 is recommended for the peak of interest.

The retention time of the test substance can be varied, if necessary, by changing the relative proportion or composition of solvents in the mobile phase. In general, an increase in the proportion of a more polar solvent will lead to a shorter retention time on a NP column and a longer retention time on a reversed-phase column.

Resolution factor (R_S)

The retention between two peaks of similar height in a chromatogram can be calculated using the following formula.

$$R_s = 1.18(t_{R2} - t_{R1})/(W_{b1} + W_{b2})$$

Where,

t_{R1} and t_{R2}= retention times or baseline distances between the point of injection and the perpendicular dropped from the maximum of each of the two peaks.

W_{b1} and W_{b2} = the respective peak widths determined at half peak height, measured in the same units and t_{R1} units as t_{R2}.

Retention factor (R_f)

The retention factor, R_f, is a quantitative indication of how far a particular compound travels in a particular solvent. The R_f value is a good indicator of whether an unknown compound and a known compound are similar, if not identical. If the R_f value for the unknown compound is close or the same as the R_f value for the known compound then the two compounds are most likely similar or identical. The retention factor, R_f, is defined as R_f = distance the solute (D_1) moves divided by the distance travelled by the solvent front (D_2).

$$R_f = D_1/D_2$$

Where,

D_1 = Distance that color travelled, measured from center of the band of color to the point where the food color was applied

D_2 = Total distance that solvent traveled.

Retention time (R_t)

Retention time is the difference in time between the point of injection and its elution from a column. Retention time is measured in minutes or seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

$$t_R = t_s + t_m$$

Where, t_S is the time of analyte spend in the stationary phase t_m is the time of analyte spend in the mobile phase

Relative retention (Rr)

The relative retention (r) is calculated using the following formula:

$$r = T_{R2}\text{-}tm/T_{R1}\text{-}tm$$

Where,

t_{R2}= retention time of the peak of interest

t_{R1}= retention time of the reference peak

t_m= retention time of an unretained component.

Peak asymmetry factor (As)

The asymmetry factor for a peak can be calculated using the following formula

$$As = W_X/2d$$

Where,

Wx= Peak width at 5% of peak height, measured from the baseline.

d = Baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as Wx. Values of As, which are >2 may lead to incorrect integration, resulting in erroneous quantitation.

The main factors that influence peak symmetry depend on retention, solvent effects, incompatibility of the solute with the mobile phase, or development of an excessive void at the inlet of the column. In reverse-phase chromatography, adsorption phenomena due to the presence of residual silanol groups in the stationary phase may lead to tailing (poor peak symmetry).

Buffers in reversed-phase liquid chromatography (RPC)

Selection of a buffered aqueous mobile phase for RPC may seem intimidating, but with an understanding of the fundamental effect of pH on retention of ionic analytes and checking some C• properties of the buffer options, a logical and reasonable choice can be derived. This selection may need to be revised by consideration of certain factors, such solubility or limitations of compatibility with the means of detection. Practical aspects of buffer preparation should also be observed for reproducible and trouble-free operations. The pH of the mobile phase will dramatically affect the retention of ionic analytes within l.5 pH units of their pKa. Thus, it can be understood why control of mobile phase pH has great utility in method development and is critical for reproducible separations of ionic analytes. Obviously, if the analyte is not ionic, mobile phase pH will not directly affect its retention

Method development guide

Guidelines for analytical method validation

"Doing a thorough method validation can be tedious, but the consequences of not doing it right are waste of time, money, and resources."

Method validation

Method validation is the process by which it is established, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended purpose[18]. It is a part of the overall validation process that also includes software validation, instrument qualification and system suitability. Typical analytical characteristics used in method validation are highlighted below. Although all analytical procedures or methods used in a regulated laboratory must be validated, this chart focuses specifically on liquid chromatography. Typical analytical characteristics used in method validation, commonly referred to as the "Eight steps of method of validation."

Performance characteristics to be considered during the validation of a quantitative method in analysis

Table 3: Performance characteristics to be considered during the validation of a quantitative method in analysis

Method parameters	Short description
Precision	Random error of the method
Repeatability	Precision measured under the best condition possible (short period and one analyst.)
Intermediate Precision	Precision measure of the within-laboratory variation due to different days, analysts, equipment's, etc.
Robustness	Capacity of a method to remain unaffected by small variations in the method parameters as could Possibly occur during the normal use of the method (pH and mobile phase composition)
Reproducibility	Precision measure determined by inter-laboratory studies
Specificity	Ability to determine the analyte in presence of other compounds
Limit of detection	Lowest sample concentration that can be detected
Limit of quantitation	Lowest sample concentration that can be quantified with suitable bias and precision
Linearity	Ability of the method to obtain test results which are proportional to the concentration in the sample
Range	Concentration interval within the method has a documented suitable performance
Stability	Absence of an influence of time on the concentration of the analyte in a sample

System suitability

- Before the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality.
- This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and Precision.
- The requirements for system suitability are usually developed after method development and validation have been completed [19].

Specificity/Selectivity

- The terms selectivity and specificity are often used interchangeably. Term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other.
- If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.
- The analyte should have no interference from other extraneous components and be well resolved from them. A representative HPLC chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined

Table 4: Flow rates for column with different internal diameter

Internal diameter of column (mm)	Standard flow rate (µL/min)
4.6	1000
2.1	200
1.0	50
0.30	4

Table 5: Acceptance limits for system suitability test

Parameter	Limit
Capacity Factor	k'>2
Injection precision	RSD <1% for n ≥5
Resolution	R_s>2
Tailing factor	A_s≤2
Theoretical plates	N>2000

Table 6: Drug profile of remdesivir

IUPAC name	2-ethylbutyl (2*S*)-2-[[[(2*R*,3*S*,4*R*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-5-cyano-3,4-dihydroxyoxolan-2-yl]methoxy-phenoxyphosphoryl] amino]propanoate
Molecular Formula	C HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"27 HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"H HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"35 HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"N HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"6 HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"O HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"8 HYPERLINK "https://pubchem.ncbi.nlm.nih.gov/"P
Molecular Weight	602.6 g/mol
Description	Remdesivir, sold under the brand name Veklury[8], HYPERLINK "https://en.wikipedia.org/wiki/Remdesivir"[9] is a broad-spectrum antiviral medication developed by the biopharmaceutical company Gilead Sciences[10]. It is administered through injection into a vein[11]. HYPERLINK "https://en.wikipedia.org/wiki/Remdesivir"[12] During the COVID-19 pandemic, remdesivir was approved or authorized for emergency use to treat COVID HYPERLINK "https://en.wikipedia.org/wiki/Coronavirus_disease_2019"19 in around 50 countries[13]. Updated guidelines from the World Health Organization in November 2020 include a conditional recommendation against the use of remdesivir for the treatment of COVID-19[14]
Half life	1 h

by recovery studies, but there are three ways to determine accuracy.

- Comparison to a reference standard
- Recovery of the analyte spiked into blank matrix, or
- Standard addition of the analyte.

Precision

Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample." A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

- Repeatability
- Intermediate precision and
- Reproducibility

Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

Linearity and range

The linearity of a method is a measure of how well a calibration plot of response against concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least-squares regression. In the resulting plot slope, intercept, and correlation coefficient provide the desired information on linearity.

Range

Difference between the greatest and the smallest values of the varieties.

Range= Largest value – smallest value.

Limit of detection (LOD)

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product[14].

LOD is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions.

With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer.

A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation (SD) of the response and the slope.

The LOD limit may be expressed as:

$$LOD = 3.3\ \sigma/S$$

Where, σ = the SD of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Limit of quantification (LOQ)

LOQ is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental[20].

- Based on visual evaluation
- Based on signal-to-noise approach
- Based on the SD of the response and the slope.

The LOQ may be expressed as:

$$LOQ = 10\ \sigma/S$$

Where, σ = the SD of the response

S = the slope of the Calibration curve.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters." A good practice is to vary, important parameters in the method, systematically and measure their effect on separation[21-23].

Ruggedness

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, and different instruments, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst[24].

Stability

To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g., 1 day, 1 week, and 1 month, depending on need). For example, the analysis of even a single sample may require 10 or more chromatographic runs to determine system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed. Therefore, a few hours of standard and sample solution stability can be required even for short (10-min) separation. When more than one sample is analyzed automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability.

Stability is defined as the capacity of a drug substance or drug product to remain within the established specifications to maintain its identify, strength, quality, and purity throughout the retest or expiration dating period[16]

The purpose of stability testing is to provide evidence on how the quality of active substances or pharmaceutical products varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light. In addition, product-related factors influence the stability.

Formulas used

Correlation coefficient (r)

When the changes in one variable are associated or followed by changes in the other, it is called correlation. The numerical measure of correlation

is called the coefficient of correlation and is defined by the relation[25].

$$r = \frac{\sum (x - x')(y - y')}{\sqrt{\sum (x - x')^2 \sum (y - y')^2}}$$

Regression equation

Regression equation= I + aC

$$a = \text{slope} = \frac{Y2 - Y1}{X2 - X1}$$

I = Intercept = regression – a C

As a percentage of mean absorbance.

SD

$$S = \sqrt{\Sigma (X - X^{!})^2 / N - 1}$$

Where X = observed values

$X^{!}$ = Arithmetic mean = ΣX/N

N = Number of deviations

For practical interpretation, it is more convenient to express "S" in terms of percent of the approximate average of the range of analysis is used in the calculation of "S." This is called co-efficient of variation (C.V) or percent relative standard deviation (%RSD)[26].

C.V OR %RSD = 100* $S/X^{!}$

Average=Sum of No. of Observations/Total No. of Observation

% RSD = SD × 100

Recovery or Practical conc= Sample area Average/ Standard Area × Std. conc.

%Recovery =Standard concentration*100/Sample concentration amount (Label C)

% Assay of Drug=s*Calculated or Sample Weight/ Label claim

LOD = 3.3 * SD of Area of Linearity/Slope

LOQ = 10. * SD of Area of Linearity/Slope.

Method development and validation

Introduction to method development

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Analytical methods should be used within good manufacturing practice (GMP)[27] and good laboratory practice (GLP)[28] environments, and must be developed using the protocols set out in the ICH guidelines[17] (Q2A and Q2B).

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, before formal validation experiments[29]. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients[30].

Need for the development of a new method

Several reasons are available for the development of a new method of analysis.

- There may not be a suitable method for a particular analyte in the specific sample matrix.

- Existing methods may be too erroneous, artifact, and/or contamination prone, or they may be unreliable (having poor accuracy or precision).
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including analyte identification or detection limits, greater accuracy or precision, or better return on investment.
- There may be a need for alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

Method development using HPLC

In method development, an attempt to select the best chromatographic conditions such as the best column, the best mobile phase, and the detection wavelength to be used for routine analysis of any drug is done. For the method development by HPLC method some information about the sample is very essential, that is, number of components present in the sample, pKa values of different components, UV-Visible Spectra of each analyte, solubility in different solvents, concentration range of each component, and nature of sample. Before method development there must be some technical information, that is, chromatography method selection according to the sample properties, the sample when analyzed with HPLC, the condition where all compounds elute in a reasonable time, optimization of HPLC method with regard to analysis time, resolution, selectivity, and sensitivity[31].

Analyte standard characterization

All the information about the analyte is gathered regarding the structure, physical and chemical properties, toxicity, purity, hygroscopicity, solubility, and stability. The availability of the 100% pure standard analyte is determined along with its storage and disposal information. If multiple components are to be analyzed in a sample matrix, the number of components to be analyzed is noted and the availability of the standard for each component is checked.

Literature search and prior methodology

The available literature is searched for all types of information related to the analyte. Availability of information regarding the synthesis, physical and chemical properties, solubility or relevant analytical methods is determined. Books, periodicals, regulatory agency compendia, such as IP, USP/NF, and BP should be referred. Chemical Abstracts Service automated/computerized literature searches also should be used.

Information pertaining to prior analytical work on the analyte has to be determined within the company and compile the available data, results, reports, memos, and publications.

Choosing a suitable method

Using the available literature and previous methodology, the methods are adapted and modified. Sample preparation and instrument conditions are adopted to make use of the latest methods and instrumentation. If no previous methods exist for the analyte in the literature, work from analogy to investigate compounds that are similar in structure and properties. Usually a compound with analytical method exists that is similar to the analyte of interest.

Optimization

Choice of method

The most commonly used chromatographic methods are NP chromatography, RP chromatography, RP ion-pair chromatography, and ion-exchange chromatography. In the selection of suitable chromatographic method for organic compounds, first reversed phase should be tried, if not successful, NP should be tried, and then RP ion-pair chromatography should be tried, ion-exchange chromatography at the end.

Choice of mobile phase

In reversed phase chromatography the selection of mobile phase is very important for the analysis of the drug. We can use acetonitrile frequently as it is suitable for the entire UV range, methanol and isopropanol are not suitable below wavelength of 210 nm, acetic acid is suitable above a wavelength of 240 nm, for the preparation of buffers, both $K_2HPO_4$and KH_2PO_4 can be used in entire UV range, freshly distilled THF is suitable for HPLC above a wave length of 240 nm, TEA is suitable above 240 nm, ammonium acetate can be used above 215 nm, EDTA can be used in entire UV range, sodium phosphate is suitable above 210 nm.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 10% organic phase in the mobile phase and organic phase concentration (Methanol and Acetonitrile) can be increased up to 100% within 20–60 min. Separation can then be optimized by changing the initial mobile phase composition according to the chromatogram obtained from preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, at what mobile composition.

Changing the polarity of the mobile phase can alter the elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, and higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in un-dissociated form. Dissociation of ionic samples may be suppressed by proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase in 5% steps of the organic phase concentrations is needed.

When separating acid or bases, buffered mobile phase is required to maintain consistency in retention time and selectivity. Buffered salts reduce peak tailing for basic compounds by effectively masking silanol groups and also reduce potential ion-exchange interactions with a protonated silanol groups. As potassium is a stronger counter ion than sodium, it provides improved results compared to sodium (Na^+). Potassium phosphate is used for preparation of buffers of various pH. If band tailing is observed for basic amphoteric compounds few drops of diluted tri ethylamine or ammonium acetate is added, for acidic or amphoteric compounds, few drops of diluted tri ethylamine or ammonium acetate is tried. For neutral compounds, the aqueous eluents used in method development is water, for weak to medium acidic compounds, 100 MM H_3PO_4 buffer of pH 2.3, for weak to medium basic or acidic compounds in ionized form 100 mM H_3PO_4 buffer of pH 4.0, 50 MM H_3PO_4 buffer of pH 7.5 are used. Unknown sample should be analyzed first with water, then with an acidic and a neutral buffer.

During mixing of the solvents in the preparation of mobile phase, the difference in partial pressure of the individual solvent at a ratio they are to be combined should be considered. The solvent system must be miscible with the previously used mobile phase, if not intermediate solvent may be used, the one that is miscible with the previous mobile phase and new mobile phase.

Choice of column

Columns being the heart of HPLC for optimum separation, stable, high performance column with good selectivity, efficiency is essential requirement for rugged and reproducible method. These characteristics are dependent on the columns manufacturer's production of good quality columns and packing materials.

Column length

- Longer columns are chosen for increased resolution.
- Shorter columns are chosen for shorter analysis time, lower back pressure, fast equilibration, and less solvent consumption.

Column internal diameter

- Wider diameter columns are chosen for greater sample loading.
- Narrow columns are chosen for more sensitivity and reduced mobile phase consumption.

Particle shape

- Columns with spherical particle shapes are preferred when lower back pressure column stability and greater efficiency is required.
- Columns with irregular particle shapes are preferred when large surface area and high capacity is required.

Particle size

- Columns with small particle size of 3–4 μm are preferred for complex mixtures with similar components. Combination of a short column (10–50 mm) with small particle size is used for fast, high resolution separations.
- Columns with larger particle size of 5–10 μm are preferred for structurally different compounds.
- Columns with large particle of 15–20 μm are used for preparative separations.

Surface area

- Columns with high surface area packing are selected for more capacity, greater resolution and longer retention.
- Columns with low surface area packing are selected for quicker equilibration time.

Carbon load

- Columns with high carbon load are chosen for greater column capacities and resolution.
- Columns with low carbon load for faster analysis time.

End capping

- Columns with end capped packing are selected to eliminate unpredictable secondary interactions with base material
- Columns with non-end capped packing are selected for selectivity differences for polar compounds by controlling secondary interactions.

Choice of detector

Detectors are eyes of the liquid chromatography system and measure the compounds after their separation on the column. Selected detector should be capable of responding to change in concentrations of all the components in the sample with adequate sensitivity even to measure trace substances. The detectors must have certain characteristics, that is, high sensitivity, higher linear dynamic range, application to most of the solutes, does not contribute to band broadening, non-destructive, and faster response.

Further optimization

After the selection of a suitable method, mobile phase, column, and detector, further optimization can be done to obtain a well-developed method.

For shorter analysis time

- Change to isocratic method. The suitable mobile phase composition is estimated from the gradient run.
- Use of shorter column, if proper resolution is obtained.

For better resolution

- Use of longer column.
- Use of stationary phase with smaller particles (3–4 μm).

For better selectivity and sensitivity

- Other station stationary phases, for example, phenyl and CN.
- pH control with ion-forming compounds
- Use of methanol or THF instead of acetonitrile.
- Detection at the absorption maximum of the substance
- All factors which lead to narrower and higher peaks as gradient elution, smaller particle, and micro bore columns.

Parameters affecting changes in chromatograph

The various parameters affecting the changes in chromatographic conditions are

- Flow rate
- Temperature
- pH
- Ion pair reagent
- Column efficiency
- Capacity factor
- Resolution
- Retention time
- Peak asymmetry.

Effect of flow rate

The efficiency of a HPLC column varies with flow rate. It is sometimes useful and readily utilized to increase the resolution. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation (less band broadening). However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase. A slower flow rate will decrease the column back pressure and a corresponding increase in the run time is observed.

Effect of temperature

Elevated temperatures decrease viscosity and increase solubility and diffusivity. Retention, peak shape, column efficiency, and total analysis time are affected by temperature because both the thermodynamics and kinetics of adsorption processes are functions of temperature. In addition, temperature control results in improved reproducibility. In liquid chromatography temperature is used in the optimization of a separation. At higher temperature, peaks will be sharper and elute earlier. System pressure is affected by temperature. The viscosity of the mobile phase decreases with increasing temperature. For example, if the HPLC system pressure is too high for a given solvent system temperature of the column may be raised to 40°C or even 60°C. Higher temperature will lead to a shorter column lifetime and some columns may not be able to tolerate 60°C. The combination of smaller diameter packing with shorter column lengths at elevated temperatures facilitates efficient and fast separations, meeting the high throughput performance requirements of the pharmaceutical industry.

Role of pH

For some preparations, the effect of changing pH is minimal. However, for acids and bases, a small change in pH is significant. Changing the pH changes the degree of ionization of molecules in solution, affecting polarity of the solution thereby changing the retention times in an HPLC separation. In a sample mixture, the retention time of the components of the mixture is also changed to different extents. Hence, it affects the degree of selectivity, where the peaks become further apart or at a particular pH they may co-elute and then the peak elution order will change. Selection of a proper buffer pH is necessary to reproducibly separate ionizable compounds by RP-HPLC. Selection of an improper pH for ionizable analytes leads to asymmetric peaks that are broad, tall or split.

During the selection of a buffer, pKa of the analyte should be considered. A buffer with 2 pH units above or below pKa of the analyte is recommended for a good peak shape. From Henderson-Hassel back equation,

$$pH = pKa + \log ([A^-]/[HA])$$

It can be determined that 99% of the analyte is in a single form, Good peak shape is possible only when analyte is in a single form.

Role of ion-pair reagent

Most of these compounds are ionic or polar; hence the use of RP-HPLC is somewhat restricted. Initially when deciding to select RP-HPLC or RP-HPLC with ion-pairing, the nature of the analyte of interest is considered. If the sample is neutral, RP-HPLC is used first; and if the sample is ionic, RP-HPLC with ion pairing is used. Thus, RP-HPLC and RP-HPLC with ion pairing are similar except that the latter consists of an ion-pair reagent in the mobile phase to improve the selectively of ionic samples. The ionic pair reagents are large

ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes.

The use of an ion-pair reagent is suggested only when separation is not adequate with RP HPLC. This is because using an ion-pair reagent introduces additional experimental parameters that need to be controlled, such as selection of a suitable ion-pair reagent to use and its concentration. Because of this added variable, RP HPLC should be utilized on any ionic analyte first before trying ion-pair RP HPLC. The approach used in RP-HPLC to separate charged analytes is ionic suppression. This technique is based on the pH adjustment of the mobile phase to result in a non-ionized analyte. However, this requires extensive method development and is only suitable for single compounds or simple mixtures where the P^{Ka}'s of the analytes lie close together.

Column efficiency (N)

The efficiency of a chromatographic column is given terms of number of theoretical plates (plate number), N

$$N = 16\left(\frac{t_r}{w}\right)^2$$

Where, t_r - retention time measured from the time of injection, w - peak width peak width obtained by drawing tangents to the sides of the Gaussian curve at the inflection points and extrapolating the tangents to intercept the baseline as in.

Efficiency of the column is also expressed as height equivalent to theoretical plate, (or plate height) HETP (or h)

$$h = \left(\frac{L}{N}\right)$$

Where, L - length of the column, N - number of theoretical plates.

Capacity factor (k')

It is measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter which specifies the extent of delay of substance to be separated.

$$k' = \left(\frac{t_r - t_m}{t_m}\right)$$

Where, t_r - retention time of the solute, t_m – retention time of the unreturned compound by the column packing. Shows capacity factor of a solute.

K' depends at stationary phase, mobile phase, temperature, and quality of column packing. For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10. If k' <1.0, the bands are in adequately separated from excessively un retained material, if k' >10 separation takes too long and bands broadened, if k' >30, satisfactory isocratic separation using present column and mobile phase is not obtained and gradient elution should be tried.

Resolution (R_s)

The ability of the column to separate two solutes. In a chromatogram it is the distance of separation of two peaks.

$$R_s = \left(\frac{t_{r2} - t_{r1}}{0.5(w_1 - w_2}\right)$$

Where, t_{r1}, t_{r2} – retention time of two immediately adjacent peaks,

w_1, w_2 – peak widths of two immediately adjacent peaks

Selectivity factor (α)/relative retention

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks separation depends on the components interaction with the stationary phase.

$$\alpha = \left(\frac{k'_b}{k'_a}\right)$$

Where,

k'_a, k'_b – capacity factors of peak a and peak b, respectively.

The value of the separation factor is always greater than unity. The separation factor is also identical to

the ratio of the corresponding distribution constants. If the capacity factor is used, the separation factor should be consistent for a given column, mobile phase composition and specified temperature, regardless of the instrument used.

Peak asymmetry (A_s)

It is also known as tailing factor. The asymmetry factor for a peak can be calculated using the following formula.

$$A_s = \left(\frac{W_{0.05}}{2f} \right)$$

Where, $W_{0.05}$ peak width at 5% height from the base line,

f – Distance between maximum and leading edge of the peak

It is also calculated from

$$A_s = \left(\frac{b}{a} \right)$$

Where, b - distance from the point at peak maxima to the trailing edge,

a – distance from the leading edge of the peak to the peak maxima (both measured at 10% height of the baseline).

Introduction to method validation

Validation is an integral part of quality assurance; it involves the systematic study of systems, facilities and processes aimed at determining whether they perform their intended functions adequately and consistently as specified. Validation in itself does not improve processes but confirms that the processes have been properly developed and are under control.

Method validation is defined as the process of proving (through scientific studies) that an analytical method is acceptable for its intended use. To ensure compliance with quality and safety standards, the United States, Europe, Japan, and other countries have published compendia, or pharmacopeias, that describe official test methods for many marketed drug products. For example, analytical methods found in USP are legally recognized analytical procedures under section 501 (b) of the Federal Food, Drug, and Cosmetic Act. For these compendia methods, USP provides regulatory guidance for method validation. In addition, validation of analytical methods is covered by the United States Code of Federal Regulations (CFR). A great deal of effort has been devoted to the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the ICH has issued guidelines for analytical method validation. The recent FDA methods validation draft guidance documents as well as U.S. both refer to ICH guidelines. The required validation parameters, also termed analytical performance characteristics or analytical figs of merit. Methods should be validated or revalidated

- Before their introduction and routine use;
- Whenever the conditions change for which the method has been validated, for example, instrument with different characteristics.
- Wherever the method is changed and the change is outside the original scope of the method.

The validation of analytical procedures is directed to the four most common types of analytical procedures: Identification tests; quantitative tests for impurities' content; limit tests for the control of impurities; and quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Method validation (ICH guidelines)

1. Accuracy,
2. Precision,
3. Repeatability,
4. Intermediate precision.
5. Specificity/Selectivity,
6. LOD,
7. LOQ,
8. Linearity,
9. Range,
10. Robustness,
11. System Suitability.

The validation terminology in ICH guidelines differ from the validation given in USP with two exceptions. Ruggedness is not included in ICH guideline and treats system suitability as a part of

method validation, whereas the USP considers it in a separate chapter.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy should be established across the specified range of the analytical procedure.

Accuracy is measured as the percentage of the analyte recovered by assay, spiking samples in a blind study.

Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100, and 120% levels of label claim as stated in the Guidelines for Submitting Samples and Analytical Data for Methods Validation

Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

The acceptance criterion for accuracy is the RSD for all the recovery values should not be more than 2.0%.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision, and reproducibility.

The precision of an analytical procedure is usually expressed as the variance, SD or coefficient of variation of a series of measurements.

The RSD for the assay of six sample preparations should not be more than 2.0%.

Specificity

Specificity is the ability to assess accurately the analyte in the presence of components which may be expected to be present in the sample matrix. Typically, these might include impurities, degrades, and matrix, it is a measure of the degree of interference from such other things such as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to a single component only.

Specificity is divided into two separate categories: identification and assay/impurity tests. For identification purpose, specificity is demonstrated by the ability to discriminate between compounds of closely related structures or comparison to a known reference standard. For assay/impurity tests, specificity is demonstrated by the resolution of the two closely eluting compounds. These compounds are usually the major component or the active ingredient and an impurity.

LOD

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample0 which can be detected but not necessarily quantities as an exact value. It is a limit test that specifies whether or not an analyte is above or below a certain value.

It is expressed as a concentration at a specified signal to noise ratio usually a 2 or 3-to-1 ratio. Two other method can also be used to determine LOD: Visual non-instrumental methods and a means of calculation: Visual non-instrumental methods may include techniques such as thin-layer chromatography (TLC) or titrations. LODs may also be calculated based on the SD of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formula:

$$LOD = 3.3\left(\frac{SD}{S}\right)$$

Where,

SD- standard deviation

S- Slope

The SD of the response can be determined based on the SD of the blank, on the residual SD of the regression line, or the SD of y-intercepts of

regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

LOQ

The LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

The ICH has recognized the 10-to-1 signal –to-noise ratio as typical, and as for LOD, lists the same two additional options that can be used to determine LOQ:

$$LOD = 10\left(\frac{SD}{S}\right)$$

Visual non-instrumental methods and a means of calculation. The calculation method is again based on the SD of the response and the slope (S) of the calibration curve according to the formula,

Where, SD- standard deviation

S- Slope

Again, the SD of the response can be determined based on the SD of the blank, on the residual SD of the regression line, or the SD of y-intercepts of regression lines. As with LOD, the method used to determine LOQ should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

Linearity and range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the (inclusive) interval between the upper and lower levels of analyte that has been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method.

A minimum of five concentration levels, along with certain minimum specified ranges are to be determined. For assay tests, the minimum specified range is 80–120% of the target concentration. For impurity tests, the minimum range is from the reporting level of each impurity to 120% of the specification.

The relationship between the concentration (in %) of drug in sample and area of should be linear in the specified range and the correlation should not be <0.9.

Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated varying method parameters such as percent organic solvent, pH, ionic strength, or temperature, and determining the effect (if any) on the results of the method.

The RSD for the assay of drugs in a sample under deliberately modified chromatographic conditions should not be more than 2.0%.

System suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as a whole. System suitability is the checking of a system to ensure system performance before or drying the analysis unknowns. Parameters such as plate count, tailing factor, resolution, and reproducibility (% RSD retention time and area for repetitive injections) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-products.

Definitions and formulas used in method validation

Mean/Average (x_i)

The average result (ā) is calculated by summing the individual results and dividing the sum by the number (n) of individual values.

$$x_i = \frac{x_1 + x_2 + x_3 ....}{n}$$

Where, x_1, x_2, x_3.= Values of individual results
n=Number of individual results

SD

It is the root mean square deviation of values from their average.

$$SD = \sqrt{\frac{\sum (x - x_i)}{n-1}}$$

Where Σ =Sum of observations
x_i=Mean or arithmetic average (Σx/n)
x=Individual observed value
$x - x_i$=Deviation of a value from the mean
n=Number of observations

RSD

It is defined as SD expressed as the percentage of mean.

$$RSD = \frac{SD}{x_i} \times 100$$

Where SD = Standard deviation
x_i = Mean or arithmetic average (Σx/n).

Correlation co-efficient (R)

The correlation coefficient is used to indicate the relationship of two random variables. It provides a measure of the strength and direction of the correlation varying from −1 to +1. Positive values indicate that the two variables are positively correlated, meaning the two variables vary in the same direction. Negative values indicate that the two variables are negatively correlated, meaning the two variables vary in the contrary direction. Values close to +1 or −1 reveal the two variables are highly related,

$$R = \frac{n\left(\sum xy\right) - \left(\sum x\right)\left(\sum y\right)}{\sqrt{\left[\sum x^2 - \left(\sum x\right)^2\right]}\sqrt{\left[\sum y^2 - \left(\sum y\right)^2\right]}}$$

Where n = number of observations
x=first value
y=second value
Σxy=sum of products of first and second value
Σx=sum of first values
Σy=sum of second values
Σx^2=sum of squares of first value
Σy^2=sum of squares of second value

Linear regression

A regression is a statistical analysis assessing the association between two variables. It is used to find the relationship between two variables.
The equation of straight line is

$$y = a + bx$$

Where b=slope
a=intercept

$$\text{Slope (b)} = [n(\Sigma xy) - (\Sigma x)(\Sigma y)]/[n(\Sigma x^2) - (\Sigma x)^2)$$

$$\text{Intercept (a)} = [(\Sigma y) - b(\Sigma x)]/n$$

Where n=number of observations
x=first value
y=second value
Σxy=sum of products of first and second value
Σx=sum of first values
Σy=sum of second values
Σx^2=sum of squares of first value
Σy^2=sum of squares of second value

Literature review of analytical methods for Remdesivir

Valeria Avataneo,[d1] Amedeo de Nicolò,[d1] Jessica Cusato,[d1] Miriam Antonucci,[d1] Alessandra Manca,[d1] Alice Palermiti,[d1] Catriona Waitt,[d2,d3] Stephen Walimbwa,[d3] Mohammed Lamorde,[d3] Giovanni di Perri,[d1,d4] and Antonio D'Avolio[d1,d], Development and validation of a UHPLC-MS/MS method for quantification of the prodrug remdesivir and its metabolite GS-441524: a tool for clinical pharmacokinetics of SARS-CoV-2/COVID-19 and Ebola virus disease:

Background: Remdesivir has received significant attention for its potential application in the treatment of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Remdesivir has already been tested for Ebola virus disease treatment and found to have activity against SARS and MERS coronaviruses. The remdesivir core contains GS-441524, which interferes with RNA-dependent RNA polymerases alone. In non-human primates, following IV administration, remdesivir is rapidly distributed into PBMCs and converted within 2 h to the active nucleoside triphosphate form, while GS-441524 is detectable

in plasma for up to 24 h. Nevertheless, remdesivir pharmacokinetics and pharmacodynamics in humans are still unexplored, highlighting the need for a precise analytical method for remdesivir and GS-441524 quantification. **Objectives:** The objectives of the study were to study the validation of a reliable UHPLC-MS/MS method for remdesivir and GS-441524 quantification in human plasma. **Methods:** Remdesivir and GS-441524 standards and QC were prepared in plasma from healthy donors. Sample preparation consisted of protein precipitation, followed by dilution and injection into the QSight 220 UHPLC-MS/MS system. Chromatographic separation was obtained through an Acquity HSS T3 1.8 µm, 2.1 × 50 mm column, with a gradient of water and acetonitrile with 0.05% formic acid. The method was validated using EMA and FDA guidelines. **Results:** Analyte stability has been evaluated and described in detail. The method successfully fulfilled the validation process and it was demonstrated that, when possible, sample thermal inactivation could be a good choice in order to improve biosafety. **Conclusions:** This method represents a useful tool for studying remdesivir and GS-441524 clinical pharmacokinetics, particularly during the current COVID-19 outbreak.

Srinivasa Rao Surabhi* and Dr Neelu Jaina, Validated stability indicating method for determination of Umifenovir- Remdesivir in presence of its degradation products: A simple stability indicating RP-HPLC method has been developed for the determination of Umifenovir and Remdesivir in its tablet dosage form. The chromatographic solution was optimized by using the standard solution. The chromatographic method was used by Zorbax SB C18 column of dimensions 150 × 4.6 mm, 3.5 µ, using isocratic elution with a mobile phase of acetonitrile and water with a 50:50 ratio was used for the chromatographic separation and was monitored at a wavelength 230 nm PDA detector with flow rate 1 ml/min. The total run time was 10 min. According to the ICH guidelines, the developed approach was validated. The calibration charts plotted were linear with a regression coefficient of R2 >0.999, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, and ruggedness were determined as a part of method validation and the results were found to be within the acceptable range. All the degradation products generated during the stress conditions are well separated and peaks have been well resolved with an acceptable retention period indicating that the proposed method was fast, easy, feasible, and affordable.

DRUG PROFILE

Drug profile of remdesivir

Uses of remdesivir

Remdesivir (Veklury) was the first drug approved by the FDA for treating the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. It is indicated for treatment of COVID-19 disease in hospitalized adults and children aged 12 years and older who weigh at least 40 kg. The broad-spectrum antiviral is a nucleotide analog prodrug.

Mechanism of action

COVID-19 is caused by the positive-sense RNA virus SARS-CoV-2. Replication of the viral genome is a key step in the infectious cycle of RNA viruses, including those of the *Filoviridae*, *Paramyxoviridae*, *Pneumoviridae*, and *Coronaviridae* families, and is carried out by viral RNA-dependent RNA polymerase (RdRp) enzymes or enzyme complexes [9,10]. For both SARS-CoV and SARS-CoV-2, the RdRp comprises nsp7, nsp8, and nsp12 subunits under physiological conditions, although functional RdRp complexes can be reassembled *in vitro* that incorporate only the nsp8 and nsp12 subunits, similar to the Middle East respiratory syndrome coronavirus (MERS-CoV)[10].

Remdesivir is a phosphoramidite prodrug of a 1'-cyano-substituted adenosine nucleotide analogue that competes with ATP for incorporation into newly synthesized viral RNA by the corresponding RdRp complex[10]. Remdesivir enters cells before being cleaved to its monophosphate form through the action of either carboxylesterase 1 or cathepsin A; it is subsequently phosphorylated by undescribed kinases to yield its active triphosphate form

remdesivir triphosphate (RDV-TP or GS-443902) [9,18]. RDV-TP is efficiently incorporated by the SARS-CoV-2 RdRp complex, with a 3.65-fold selectivity for RDV-TP over endogenous ATP[10,18]. Unlike some nucleoside analogs, remdesivir provides a free 3'-hydroxyl group that allows for continued chain elongation[10,18]. However, modeling and *in vitro* experiments suggest that at $i + 4$ (corresponding to the position for the incorporation of the fourth nucleotide following RDV-TP incorporation), the 1'-cyano group of remdesivir sterically clashes with Ser-861 of the RdRp, preventing further enzyme translocation and terminating replication at position $i + 3$. This mechanism was essentially identical between SARS-CoV, SARS-CoV-2, and MERS-CoV, and genomic comparisons reveal that Ser-861 is conserved across alpha-, beta-, and delta-coronaviruses, suggesting remdesivir may possess broad antiviral activity[10].

Considerations for the use of nucleotide analogs such as remdesivir include the possible accumulation of resistance mutations. Excision of analogs through the 3'-5' exonuclease (ExoN) activity of replication complexes, mediated in SARS-CoV by the nsp14 subunit, is of possible concern[10]. Murine hepatitis viruses (MHVs) engineered to lack ExoN activity are approximately 4-fold more susceptible to remdesivir, supporting the proposed mechanism of action[3]. However, the relatively mild benefit of ExoN activity to remdesivir resistance is proposed to involve its delayed chain termination mechanism, whereby additional endogenous nucleotides are incorporated following RDV-TP[10]. In addition, serial passage of MHV in increasing concentrations of the remdesivir parent molecule GS-441524 led to the development of resistance mutations F476L and V553L, which maintain activity when transferred to SARS-CoV. However, these mutant viruses are less fit than wild-type in both competition assays and *in vivo* in the absence of selective pressure[3]. To date, no clinical data on SARS-CoV-2 resistance to remdesivir have been described[18].

Absorption

Remdesivir is absorbed quickly; maximal plasma concentrations following a single 30-min intravenous infusion are reached within 0.67–0.68 h (T_{max}). Repeated dosing yields a C_{max} (coefficient of variation as a percent) of 2229 (19.2) ng/mL and an AUC_{tau} of 1585 (16.6) ng*h/mL[18].

Remdesivir metabolite GS-441524 has measured values: T_{max} 1.51–2.00 h, C_{max} 145 (19.3) ng/mL, AUC_{tau} 2229 (18.4) ng*h/mL, and C_{trough} 69.2 (18.2) ng/mL. Another metabolite, GS-704277, has measured values: T_{max} 0.75 h, C_{max} 246 (33.9) ng/mL, AUC_{tau} 462 (31.4) ng*h/mL, and an undetermined C_{trough} [18].

A 10 mg/kg intravenous dose given to cynomolgus monkeys distributes to the testes, epididymis, eyes, and brain within 4 h[1].

Contraindications

VEKLURY is contraindicated in patients with a history of clinically significant hypersensitivity reactions to VEKLURY or any components of the product.

EXPERIMENT [FIGURES 1-60]

HPLC simultaneous method development for remdesivir equipment

Table 7: List of Apparatus used in HPLC

S. no	Name	Model	Manufacturer
1	HPLC	Acquity	Waters
2	pH meter	-	Eutech
3	Weighing balance	-	Sartouris
4	UV/VIS spectrophotometer	-	UV-1700
5	Pipettes, beakers and Burettes	-	Borosil
6	Ultra sonicator	UCA 701	Unichrome
7	Pump	Isocratic model	--

HPLC: High performance liquid chromatography

Reagents and chemicals

Table 8: List of chemicals used in HPLC method

S. no	Name	Grade	Manufacturer
1.	Acetonitrile	HPLC	Rankem
2.	Water (Milli Q)	HPLC	In house production
3.	Ortho Phosphoric acid	HPLC	Analytical reagents
4.	Methanol	HPLC	Rankem

HPLC: High-performance liquid chromatography

Determination of working wavelength (λ_{max})

In estimation of the drug isobestic wavelength was used. Isobestic point is the wavelength where the molar absorptivity is the same for the substances that are inter convertible. So this wavelength was used in estimation of drug accurately.

The wavelength of maximum absorption of the solution of the drug in mixture of Acetonitrile and 0.1% Orthophthaldehyde (OPA) (70:30) was scanned using PDA Detector within the wavelength region of 200–400 nm against Acetonitrile and 0.1% OPA (70:30) as blank. The absorption curve shows isobestic point at 246 nm. Thus, 246 nm was selected as detector wavelength for the HPLC chromatographic method.

Chromatographic conditions

During the selection of chromatographic conditions, numbers of trails were carried out and the best trail was selected for optimized method.

Preparation of standard stock solution

Accurately weigh and transfer 50 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (50 ppm of Remdesivir).

Sample solution preparation

Accurately weighed and transfer equivalent to 50 mg of Remdesivir sample into a 100mL clean dry volumetric flask add Diluent and sonicate it up to 30 min to dissolve, and centrifuge for 30 min to dissolve it completely and make volume up to the mark with the same solvent. Then, it is filtered through 0.45 µ Injection filter (Stock solution). Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (50 ppm of Remdesivir.)

Trails in optimization of chromatographic condition

The Remdesivir peak was observed at 2.423 min with peak area 2310254, tailing factor 0.60. This trial was optimized.

General preparations

Preparation of 0.1% OPA buffer solution

Take 1 ml of OPA dissolved in 1 l of HPLC grade water. Filter through 0.45 µ nylon filter.

Preparation of mobile phase

Mobile phase was prepared by mixing 0.1% OPA and ACN taken in the ratio 30:70. It was filtered through 0.45 µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Chromatographic condition

Use Waters Acquity HPLC.
Column: Luna phenyl hexyl (250 × 4.6 mm, 5 µm)
Mobile phase ratio: Acetonitrile and 0.1% OPA 70:30
Detection wavelength: 246 nm
Flow rate: 1 ml/min
Injection volume: 10 µl
Run time: 5 min.

Preparation of diluent

Use mobile phase as a diluent.

Preparation of standard stock solution

Accurately weigh and transfer 50 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (50 ppm of Remdesivir).

Sample solution preparation

Accurately weighed and transfer equivalent to 50 mg of Remdesivir sample into a 100 mL clean dry volumetric

Table 9: TRIAL-1 chromatographic conditions

Column	X-Bridge phenyl 150×4.6 mm, 3.5 µ
Mobile phase ratio	ACN+ Water (80+20)
Detection wavelength	200–400 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	15 min
Observation:	Splitting of the peak is observed

Table 10: TRIAL-2 chromatographic conditions

Column	X-Bridge phenyl 150×4.6 mm, 3.5 µ
Mobile phase ratio	ACN+Water (70+30)
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	6 min
Observation	Plate count not within the limit

Table 11: TRIAL-3 chromatographic conditions

Column	Symmetry C18 150×4.6 mm, 3.5 µ
Mobile phase ratio	ACN+Water (60+40)
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	15 min
Observation	Tailing is not within the limit

Table 12: TRIAL-4 chromatographic conditions

Column	Symmetry C18 150×4.6 mm, 3.5 µ
Mobile phase ratio	ACN and 0.1% TFA 50:50
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	7 min
Observation	Two peaks are observed

Table 13: TRIAL-5 chromatographic conditions

Column	Symmetry C18 150×4.6 mm, 3.5 µ
Mobile phase ratio	ACN and 0.1% TFA 60:40
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	3 min
Observation	Peak shape is not good

Table 14: TRIAL-6 chromatographic conditions

Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Mobile phase ratio	ACN and 0.1% TFA 70:30
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	7 min
Observation	Tailing is not within the limit

flask add diluent and sonicate it up to 30 min to dissolve, and centrifuge for 30 min to dissolve it completely and make volume up to the mark with the same solvent. Then, it is filtered through 0.45 µ Injection filter (Stock solution). Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (50 ppm of Remdesivir).

Procedure

Inject 10 L of the standard, sample into the chromatographic system and measure the areas for Remdesivir peak and calculate the % Assay using the formulae.

SYSTEM SUITABILITY

Tailing factor for the peak due to Remdesivir in Standard solution should not be more than 2.0
Theoretical plates for the Remdesivir peak in Standard solution should not be <2000.

Formula for assay:

Where: AT= average area counts of test (sample) preparation.
AS= average area counts of standard preparation.
WS= Weight of working standard taken in mg.
DS= Dilution of working standard in ml.
DT= Dilution of test (sample) in ml.
WT= Weight of test (sample) taken in mg.
P= Percentage purity of working standard
LC= Label Claim mg/ml.

METHOD VALIDATION SUMMARY

Specificity

Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were

recorded. The chromatogram of blank shows no response at the retention times of drugs which confirms the response of drug was specific.

LINEARITY

Table 15: TRIAL-7 chromatographic conditions

Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Mobile phase ratio	ACN and 0.1% OPA 40:60
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	6 min
Observation	Broad peak is obtained

Table 16: TRIAL-8 chromatographic conditions

Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Mobile phase ratio	ACN and 0.1% OPA 50:50
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	19 min
Observation	Less retention time is obtained

Table 17: TRIAL-9 chromatographic conditions

Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Mobile phase ratio	ACN and 0.1% OPA 60:40
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	11 min
Observation	Unknown peak is formed

Table 18: TRIAL-10 optimized chromatographic conditions

Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Mobile phase ratio	ACN and 0.1% OPA 70:30
Detection wavelength	246 nm
Flow rate	1 ml/min
Injection volume	10 µl
Run time	5 min
Observation	Suitable method for validation

Preparation of stock solution

Accurately weigh and transfer 50 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Preparation of level – I (12.5 ppm of remdesivir)

1.25 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of level – II (25 ppm of remdesivir)

2.5 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of level – III (37.5 ppm of remdesivir)

3.75 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of level – IV (50 ppm of remdesivir)

5 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Table 20: Optimized chromatographic conditions

Parameters	Observation
Instrument used	Waters Alliance e-2695 HPLC
Injection volume	10µl
Mobile Phase	ACN and 0.1% OPA 70:30
Column	Luna phenyl hexyl (250×4.6 mm, 5 µm)
Detection Wave Length	246 nm
Flow Rate	1 mL/min
Runtime	5 min
Temperature	Ambient (25°C)
Mode of separation	Isocratic mode

HPLC: High-performance liquid chromatography

Table 19: Results for (Optimized trail)

	Name	RT	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1	Remdesivir	2.423	2,310,254	100		0.60	6500

Preparation of level –V (62.5 ppm of remdesivir)

6.25 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of level – VI (75 ppm of remdesivir)

7.5 ml of above stock solutions has taken in different 50 ml of volumetric flasks, dilute up to the mark with diluent.

Procedure

Inject each level into the HPLC chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Range

The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that has been demonstrated with precision, accuracy and linearity

Acceptance criteria

Correlation coefficient should be not <0.999.

Preparation accuracy sample solutions

For preparation of 50% solution (with respect to target assay concentration)
Accurately weigh and transfer 25 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (25 ppm of Remdesivir).

For preparation of 100% solution (with respect to target assay concentration)
Accurately weigh and transfer 50 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).
Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (50 ppm of Remdesivir).

For preparation of 150% solution (with respect to target assay concentration)
Accurately weigh and transfer 75 mg of Remdesivir working standard into a 100 ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).
Further pipette 5 ml of the above stock solutions into a 50 ml volumetric flask and dilute up to the mark with diluent (75 ppm of Remdesivir).

Procedure

Inject the standard solution, Accuracy - 50%, Accuracy - 100%, and Accuracy - 150% solutions.

Acceptance criteria

The % Recovery for each level should be between 98.0 and 102.0%

Precision

Precision is the degree of repeatability of an analytical method under normal operation conditions. Precision is of three types

1. System precision
2. Method precision
3. Intermediate precision (a) intraday precision, and (b) interday precision

System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations are measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample 6 times and calculate the % RSD.
The precision of the instrument was checked by repeatedly injecting (n = 6) solutions of 50 ppm of Remdesivir.

Acceptance criteria

The % RSD for the absorbance of six replicate injections results should not be more than 2%.

ROBUSTNESS

As part of the Robustness, deliberate change in the flow rate, mobile phase composition, temperature variation was made to evaluate the impact on the method.
A. The flow rate was varied at 0.8–1.2 ml/min.
Standard solution 50 ppm of Remdesivir was prepared and analyzed using the varied flow rates along with method flow rate.
On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence, it indicates that the method is robust even by change in the flow rate ±20%.
B. The variation of Organic Phase ratio.
Standard solution of 50 ppm of Remdesivir was prepared and analyzed using the varied in mobile phase ratio.

LOD and LOQ

The LOD and LOQ of the drug carry were calculated using the following equation as per ICH guidelines.
$LOD = 3.3 \times \sigma/S$
$LOQ = 10 \times \sigma/S$
LOD for Remdesivir was found to be 0.063 μg/mL and LOQ for Remdesivir was found to be 0.206 μg/ml.

DEGRADATION STUDIES

Preparation of stock

Accurately weigh and transfer weight equivalent to 50 mg of Remdesivir sample into a 100 ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Acid degradation

Pipette 5 ml of above solution into a 50 ml volumetric flask and 3 ml of 1N HCl was added. Then, the volumetric flask was kept at 60°C for 6 h and then neutralized with 1 N NaOH and make up to 50 ml with diluent. Filter the solution with 0.22 μ syringe filters and place in vials.

Alkali degradation

Pipette 5 ml of above solution into a 50 ml volumetric flask and add 3 ml of 1 N NaOH was added. Then, the volumetric flask was kept at 60°C for 6 h and then neutralized with 1 N HCl and make up to 50 ml with diluent. Filter the solution with 0.22 μ syringe filters and place in vials.

Thermal induced degradation

Remdesivir sample was taken in Petri dish and kept in Hot air oven at 110°C for 24 h. Then, the sample was taken and diluted with diluents and injected into HPLC and analyzed.

Oxidative degradation

Pipette 5 ml above stock solution into a 50 ml volumetric flask, 1 ml of 30% of hydrogen peroxide added in 50 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min filter the solution with 0.45 μ syringe filters and place in vials.

Reduction degradation

Pipette 5 ml above stock solution into a 50 ml volumetric flask, 1 ml of 30% of sodium bisulfate

solution added in 50 ml of volumetric flask and the volume was made up to the mark with diluents. The volumetric flask was then kept at room temperature for 15 min filter the solution with 0.45 µ syringe filters and place in vials.

RESULTS AND DISCUSSION [TABLES 1-30]

HPLC method

Determination of Working Wavelength (λ_{max})

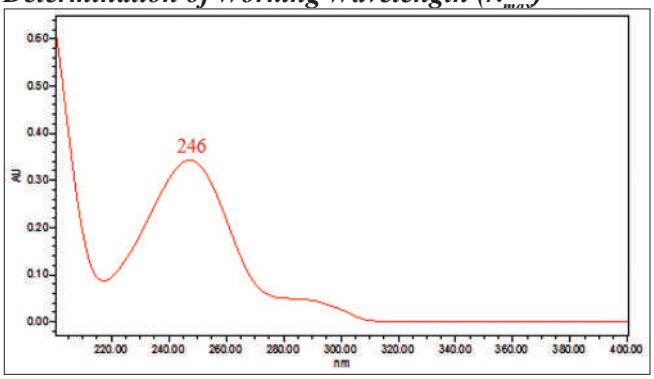
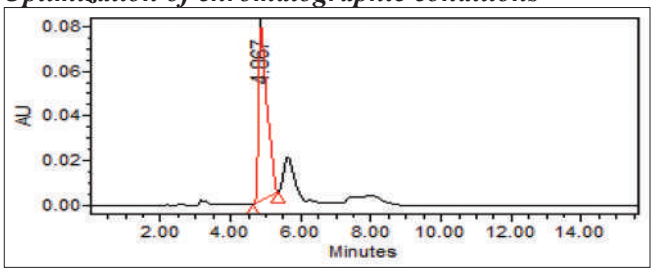


Figure 13: PDA - spectrum of remdesivir

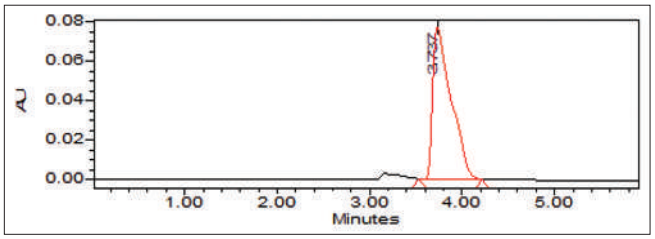
Optimization of chromatographic conditions



	Name	Retention time	Area	% Area	USP tailing	USP plate count
1		4.867	546,321	100.02	4.56	8563

Figure 14: Chromatogram of Trial-1

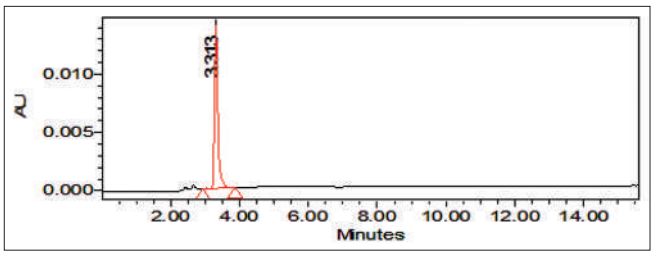
Trial-2



Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
	3.737	581,263	100.23		2.12	1382

Figure 15: Chromatogram of Trial-2

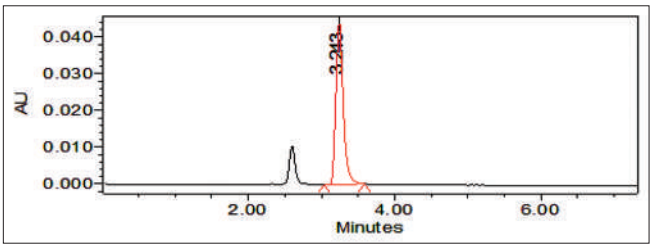
Trial-3



	Name	Retention Time	Area	% Area	USP Tailing	USP Plate Count
1		3.313	1,028,546	902,541	3.62	1888

Figure 16: Chromatogram of Trial-3

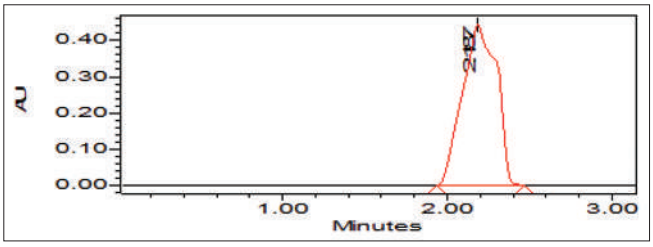
Trial-4



	Name	Retention Time	Area	% Area	USP Tailing	USP Plate Count
1		3.243	254,1263	99.86	1.16	2422

Figure 17: Chromatogram of Trial-4

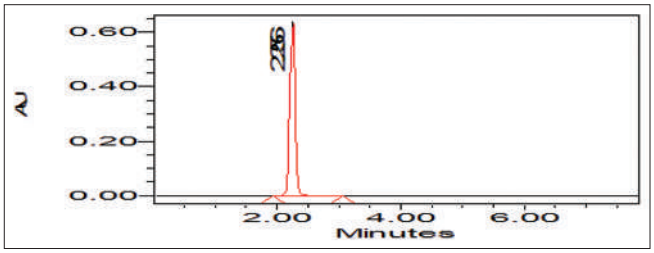
Trial-5



	Name	Retention Time	Area	% Area	USP Tailing	USP Plate Count
1		2.187	6429928	100.00	3.58	1452

Figure 18: Chromatogram of Trial-5

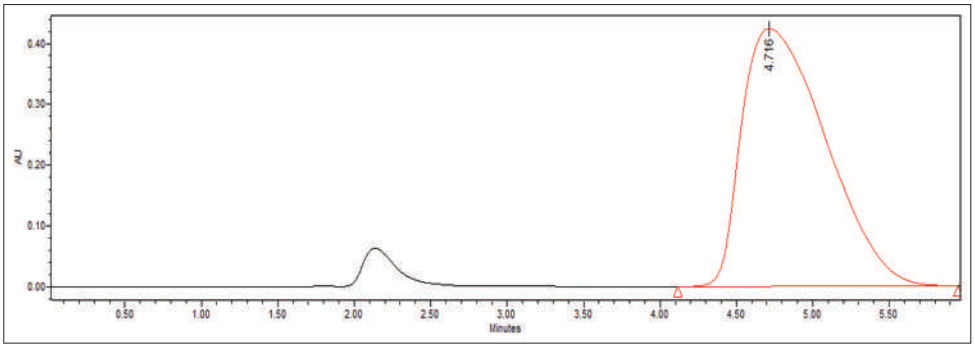
Trial-6



	Name	Retention time	Area	% Area	USP tailing	USP plate count
1		2.256	84,991	100.00	5.28	1452

Figure 19: Chromatogram of Trial-6

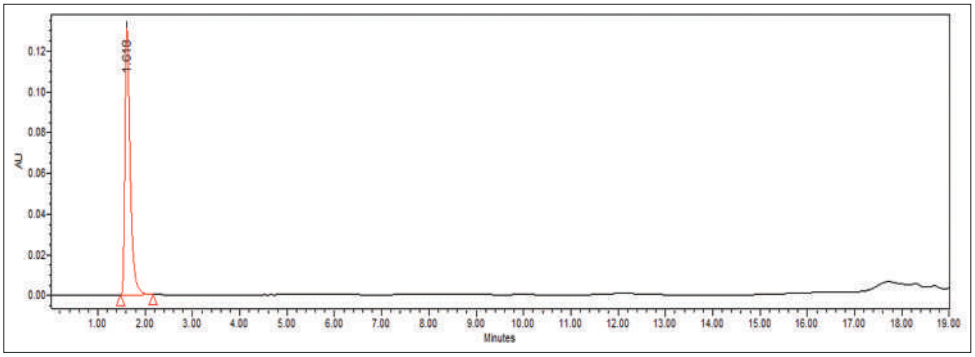
Trial-7



	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		4.716	16,217,023	100.00		1.72	355

Figure 20: Chromatogram of Trial-7

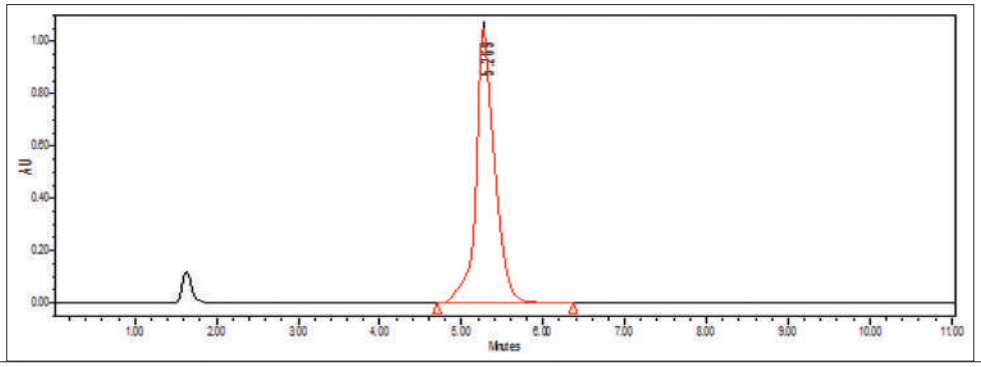
Trial-8



	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.618	1,008,168	100.00		1.58	1088

Figure 21: Chromatogram of Trial-8

Trial-9



	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		5.269	16,567,807	100.00		1.05	1452

Figure 22: Chromatogram of Trial-9

Trial-10 (optimized chromatogram)

Observation: All the parameters are within the limit this method is suitable for validation.

System suitability

All the system suitability parameters were within the range and satisfactory as per the ICH guidelines.

Acceptance criteria

According to the ICH guidelines plate count should be more than 2000, tailing factor should be <2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Assay

Table 21: System suitability parameters for Remdesivir

S. no	Parameter	Remdesivir
1	Retention time	2.423
2	Plate count	6523
3	Tailing factor	0.65
4	Resolution	----
5	%RSD	0.92

ANALYTICAL METHOD VALIDATION (HPLC)

The method was validated for its linearity range, accuracy, precision, and specificity. Method validation was carried out as per the ICH guidelines.

Specificity

Discussion

Retention times of Remdesivir were 2.422 min. We did not found and interfering peaks in blank and placebo at retention times of these drugs in this method. Hence, this method was said to be specific.

Linearity

Table 23: Results of linearity for remdesivir

S. no	Remdesivir	
	Conc. (µg/ml)	Peak area
1	12.5	614872
2	25	1255648
3	37.5	1835698
4	50	2369542
5	62.5	2932159
6	75	3463147
Regression equation	y=46108.3 x+52519.18	
Slope	79587.86	
Intercept	17504.83	
R^2	0.9994	

PRECISION

System precision

Table 24: System precision table of remdesivir

S. No	Concentration remdesivir (µg/ml)	Area of remdesivir
1.	50	2310254
2.	50	2345158
3.	50	2365982
4.	50	2358421
5.	50	2326584
6.	50	2325607
Mean		2338668
S.D		21458.443
%RSD		0.92

Discussion

From a single volumetric flask of working standard solution, six injections were given and the obtained areas were mentioned above. Average area, SD, and % RSD were calculated for two drugs. % RSD obtained as 0.92 for Remdesivir. As the limit of precision was <"2," the system precision was passed in this method.

Repeatability

Table 25: Method precision for remdesivir by HPLC method

S. No.	Area for remdesivir
1	2,366,548
2	2,321,548
3	2,342,156

Table 22: Assay of remdesivir

Brand	Drug	Area	Average sample area	Std. wt	Sample wt.	Label amount (mg)	Std purity	Amount found (µg/ml)	% assay
-	Remdesivir	2356842 2324175	2340508	50	10	5	99.9	50.2	100.1

4	2,375,451
5	2,306,541
6	2,329,854
Average	2,340,349
Standard deviation	26564.66
%RSD	1.13

HPLC: High-performance liquid chromatography

Acceptance criteria

The % RSD for the area of six standard injections results should not be more than 2%**.**

Intermediate precision (Day_Day precision)

Table 26: Intermediate precision (Day variation) for remdesivir by HPLC method

Injection	Area for remdesivir
1	2,310,214
2	2,337,248
3	2,352,445
4	2,374,892
5	2,320,158
6	2,368,501
Average	2,343,909
Standard deviation	26005.41
%RSD	1.10

HPLC: High performance liquid chromatography

Acceptance criteria

The % RSD for the area of six standard injections results should not be more than 2%.

Accuracy

Discussion

Three levels of accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.33% for Remdesivir, respectively.

LOD and LOQ (µg/ml)

DEGRADATION STUDIES

SUMMARY

An attempt has been made to develop a validated stability indicating RP-HPLC method for the estimation of Remdesivir. Literature survey revealed that many analytical methods have been reported individually or in combination with other drugs. However, no method was reported for estimation of drug by HPLC method.

The whole paper gives the general information on RP-HPLC and method development, General information on forced degradation studies and stress conditions such as acid, base, peroxide, thermal,

Table 27: Accuracy results of Remdesivir by HPLC method

%Concentration (at specification Level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean %recovery
50	1,185,458	25	25.34	101.4	100.33
100	2,352,146	50	50.29	100.9	
150	3,463,214	75	74.04	98.7	

HPLC: High-performance liquid chromatography

Table 28: Robustness results of remdesivir by-HPLC

Parameter	Remdesivir					
	Condition	Retention time (min)	Peak area	Resolution	Tailing	Plate count
Flow rate change (mL/min)	Less flow (0.8 ml)	2.690	2,560,105		0.68	6694
	Actual flow (1 ml)	2.423	2,310,254		1.00	6500
	More flow (1.2 ml)	2.192	2,062,279		0.95	6657
Organic phase change	Less Org (63:37)	2.658	2,499,870		1.12	6651
	Actual (70:30)	2.425	2,345,158		0.99	6589
	More Org (77:23)	2.261	2,262,129		1.23	6826

HPLC: High-performance liquid chromatography

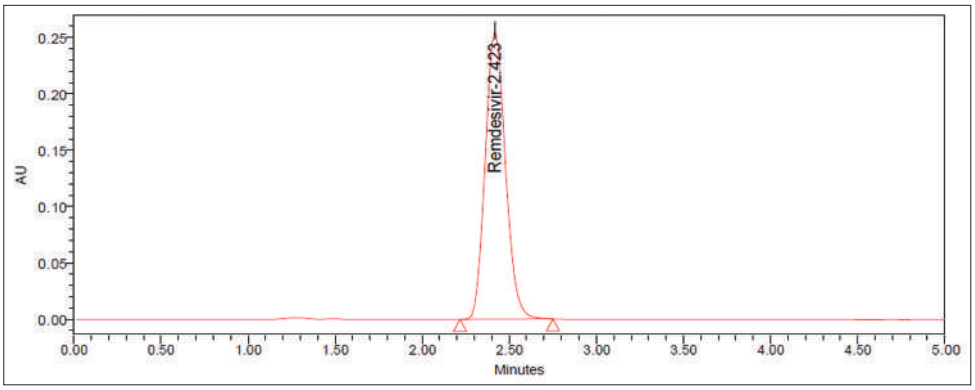


Figure 23: Optimized chromatogram -Trial-10

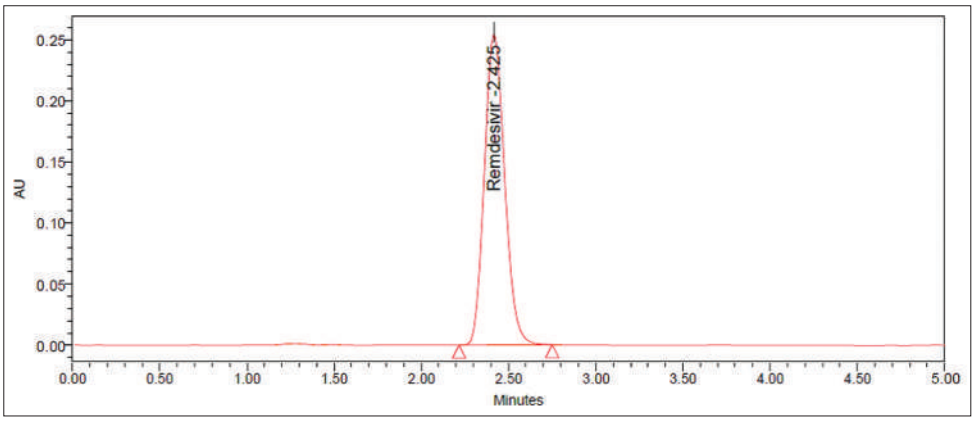


Figure 24: Chromatogram of standard

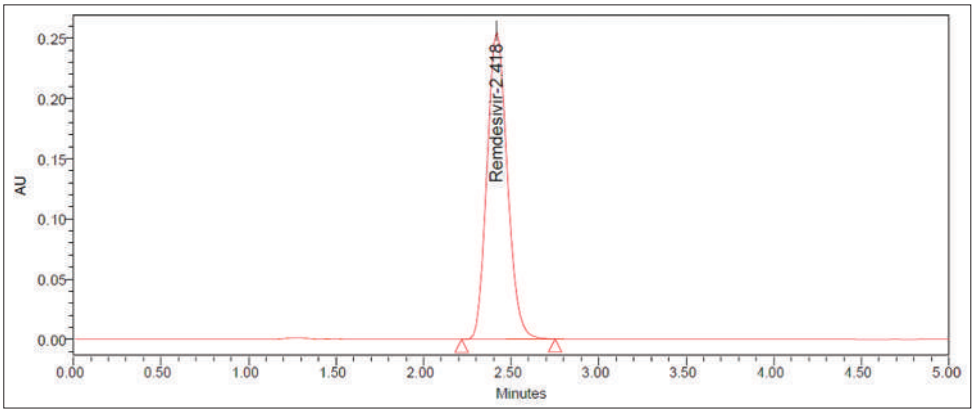


Figure 25: Chromatogram of Assay-1

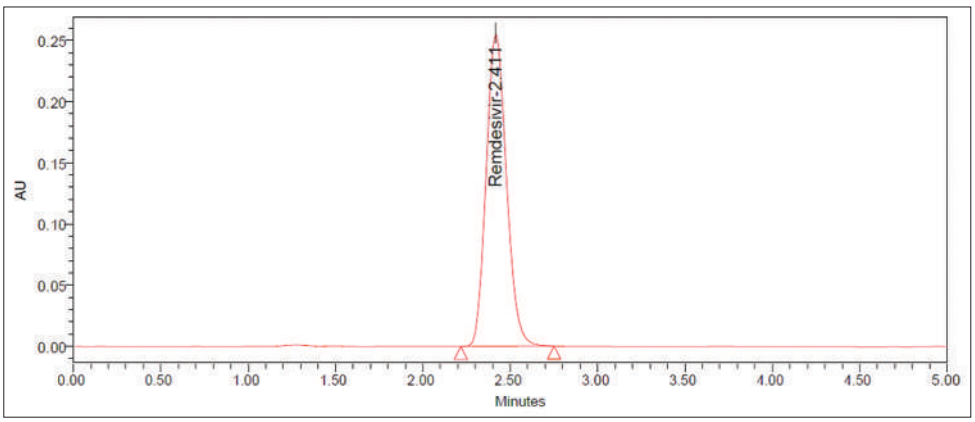


Figure 26: Chromatogram of Assay-2

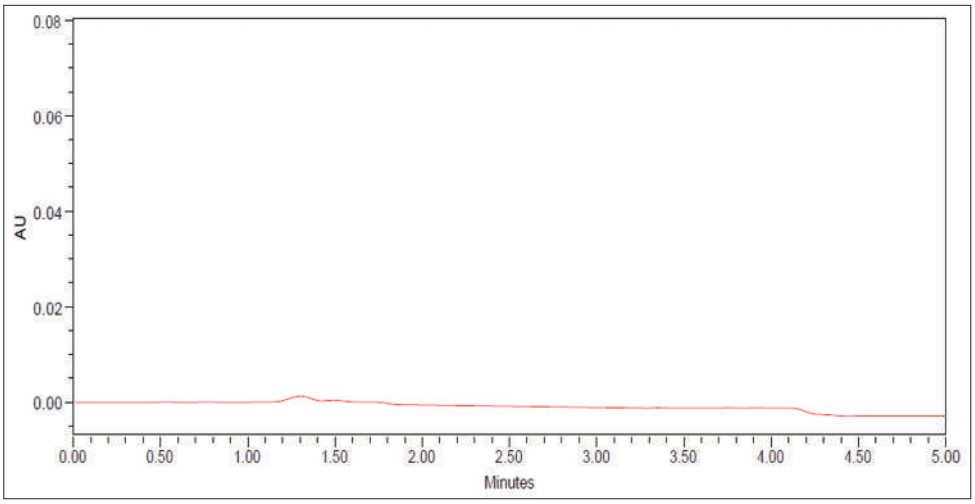


Figure 27: Chromatogram of blank

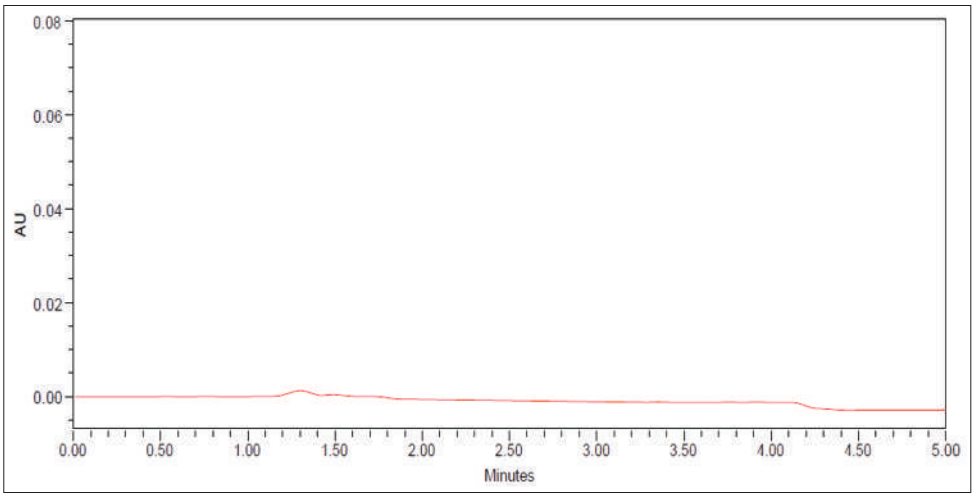


Figure 28: Chromatogram of placebo

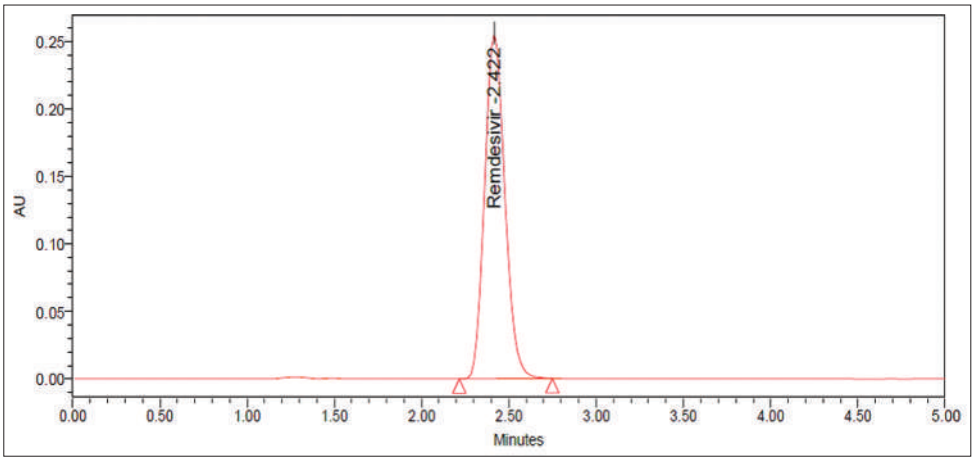


Figure 29: Optimized chromatogram

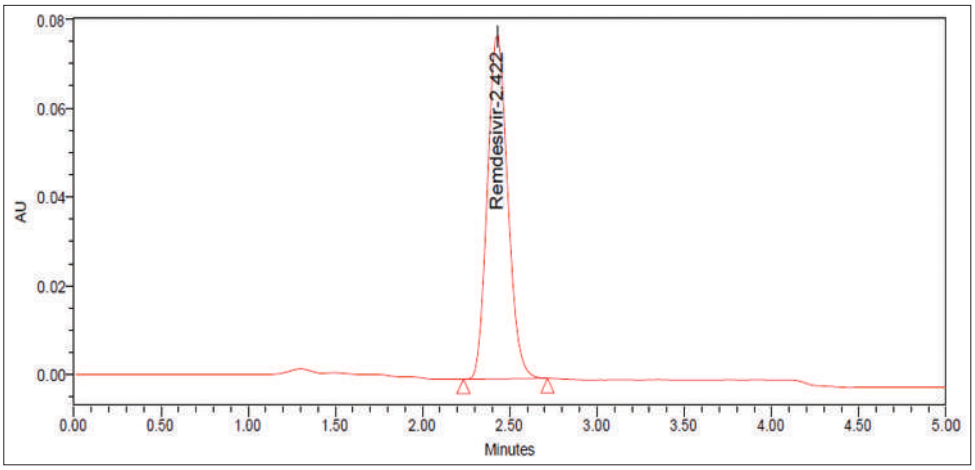


Figure 30: Chromatogram of linearity - 25%

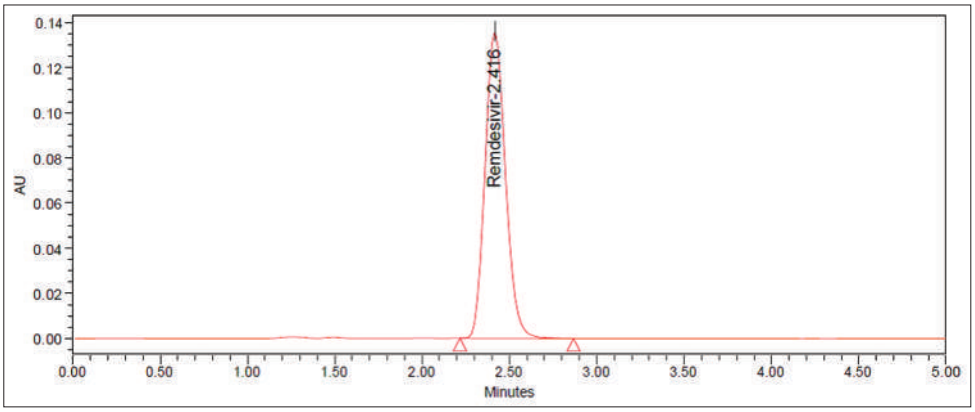


Figure 31: Chromatogram of linearity - 50%

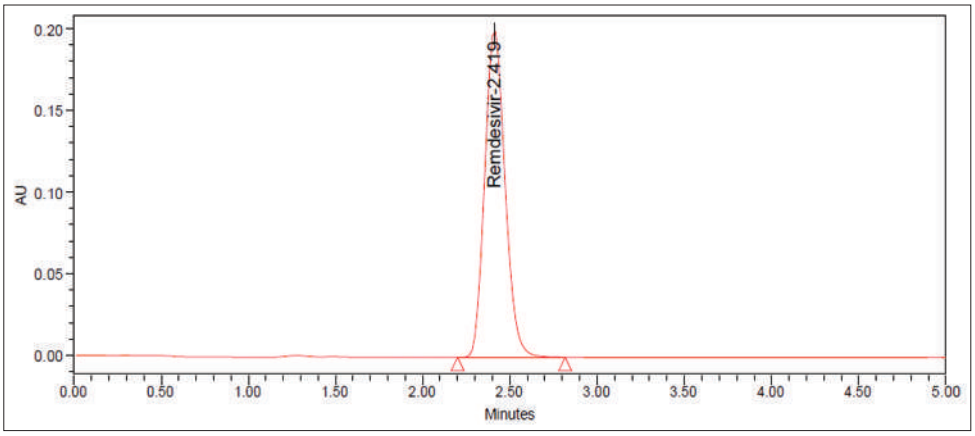


Figure 32: Chromatogram of linearity - 75%

and UV. Discusses about drug profiles and official status of selected drug, that is, Remdesivir, explain in detail the previous literature available for drugs used for developed research work, and give in detail about the aim, objective and plan of the proposed work by using selected drugs. It includes Stability Indicating RP-HPLC Method Development and Validation for Estimation of Remdesivir in Bulk and their Pharmaceutical dosage form. Using Waters alliance HPLC system, Quaternary gradient pump of e2695 series equipped with a auto sampler injector with 10 µl is injected eluted with the mobile phase containing 0.1% Ortho phosphoric acid and Acetonitrile in the ratio of 30:70 v/v which is pumped at a flow rate of 1ml/min and detected by UV detector at 246 nm. The peak of Remdesivir

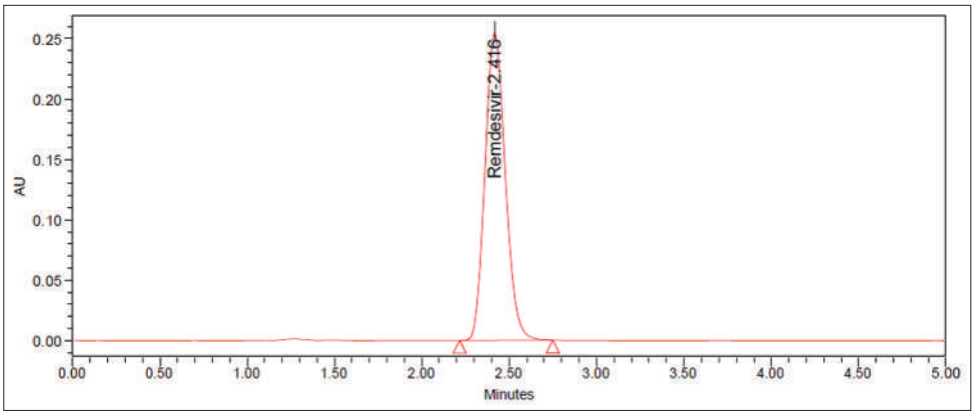


Figure 33: Chromatogram of linearity - 100%

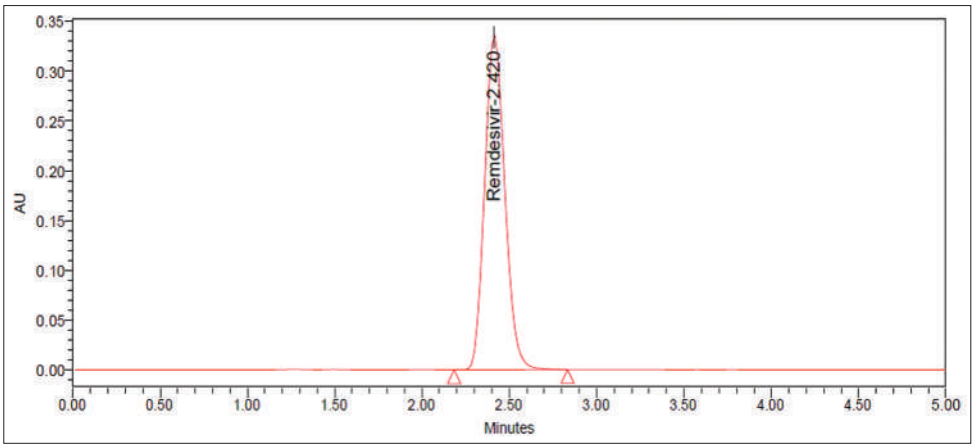


Figure 34: Chromatogram of linearity - 125%

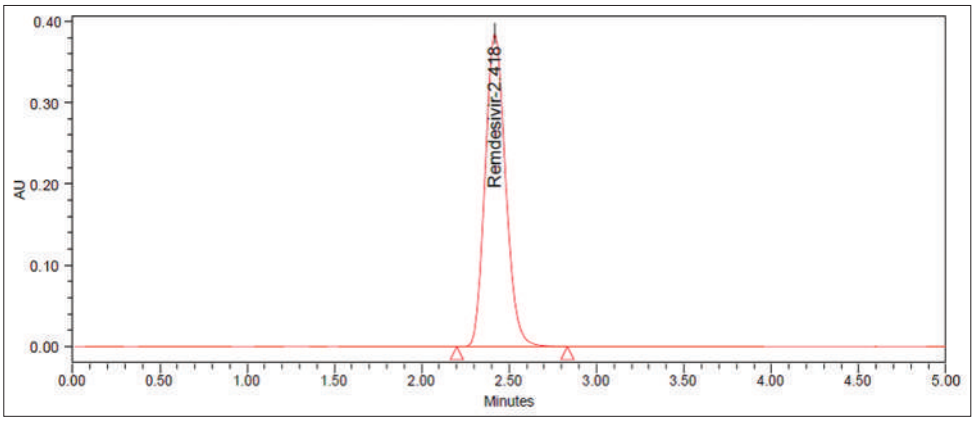


Figure 35: Chromatogram of linearity - 150%

was eluted at retention times of 2.423 min. In this proposed HPLC method for the selected drugs showed good linearity. Results for the recoveries of selected drug were found to be within limits (98–

Table 29: Sensitivity parameters (LOD and LOQ) by HPLC

Name of drug	LOD (µg/ml)	LOQ (µg/ml)
Remdesivir	0.063	0.206

HPLC: High-performance liquid chromatography

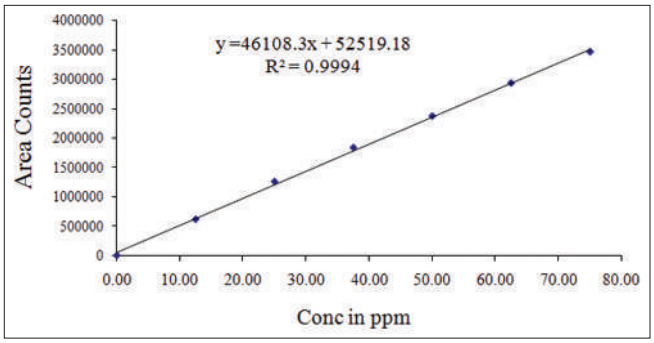


Figure 36: Calibration curve for remdesivir at 246 nm

Table 30: Forced degradation results for remdesivir

Results: % degradation results	Remdesivir	
	Area	% Degradation
Control	2,341,485	0
Acid	2,015,297	13.9
Alkali	2,092,170	10.6
Peroxide	1,982,437	15.3
Reduction	1,996,498	14.7
Thermal	1,879,123	19.7
Photo	1,943,628	17

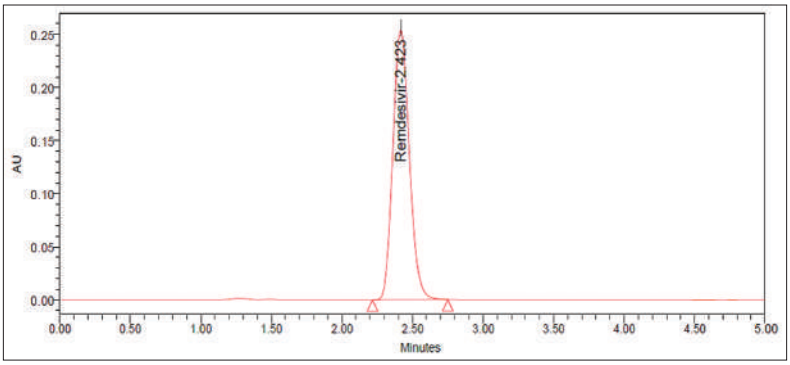


Figure 37: System precision chromatogram-1

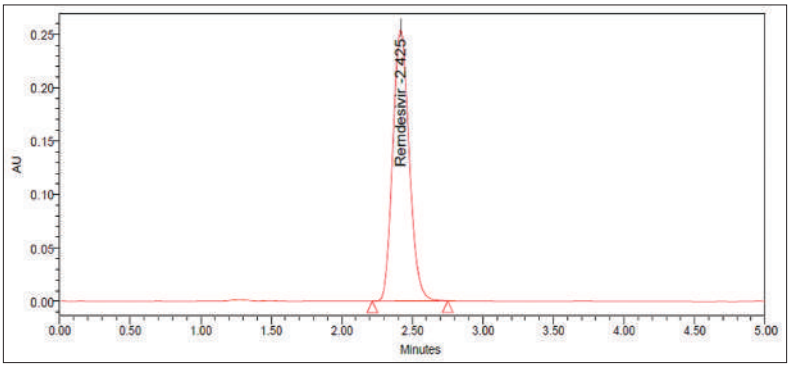


Figure 38: System precision chromatogram-2

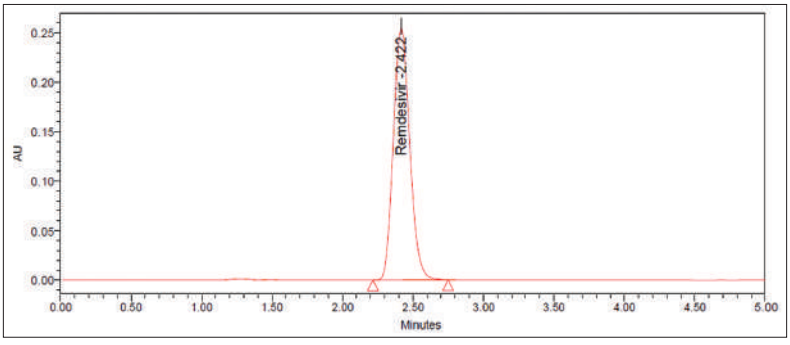


Figure 39: System precision chromatogram-3

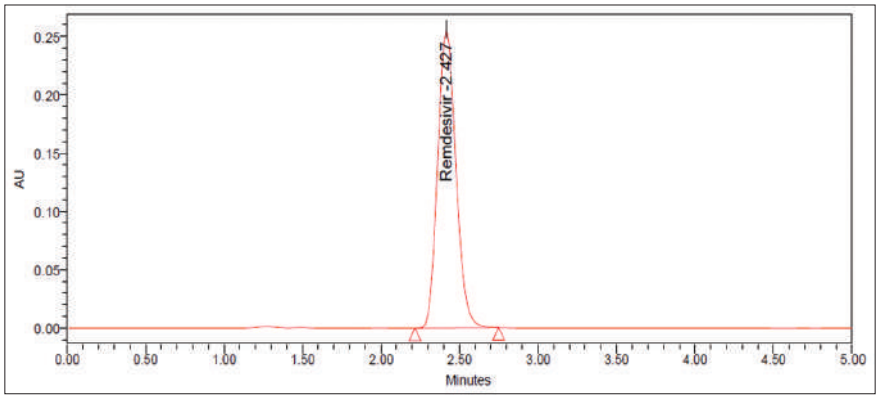


Figure 40: System precision chromatogram-4

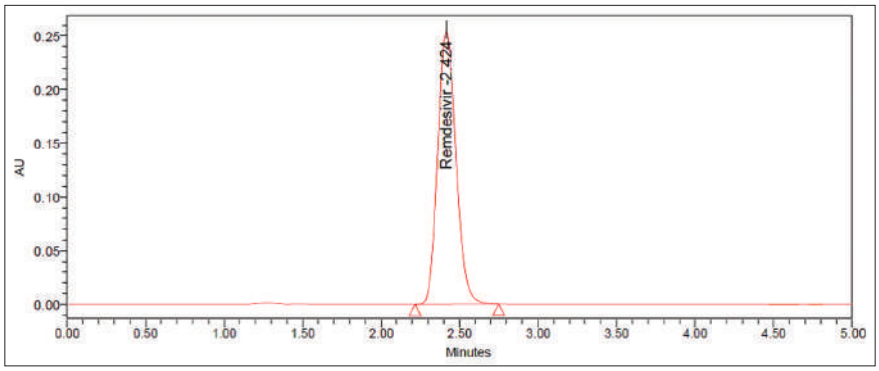


Figure 41: System precision chromatogram-5

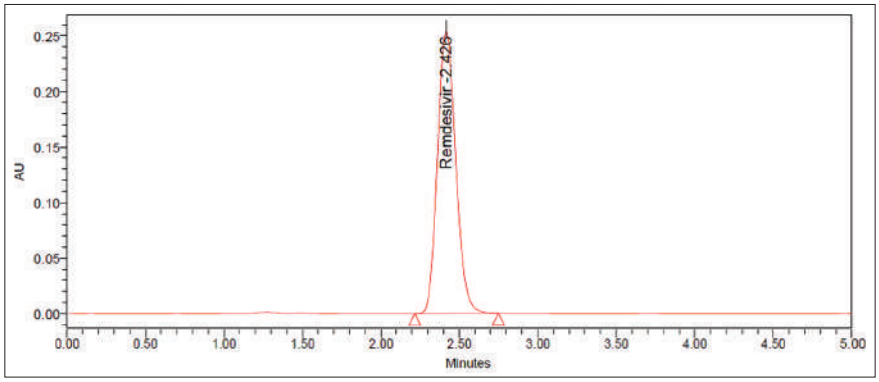


Figure 42: System precision chromatogram-6

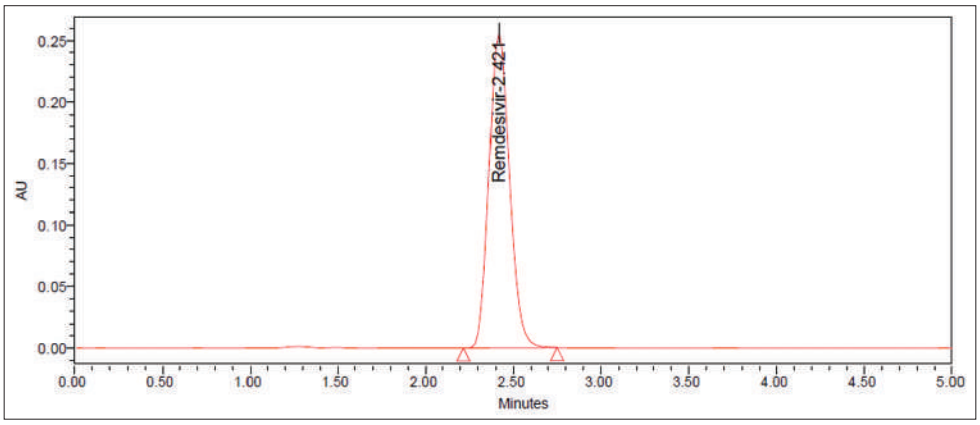


Figure 43: Repeatability chromatogram

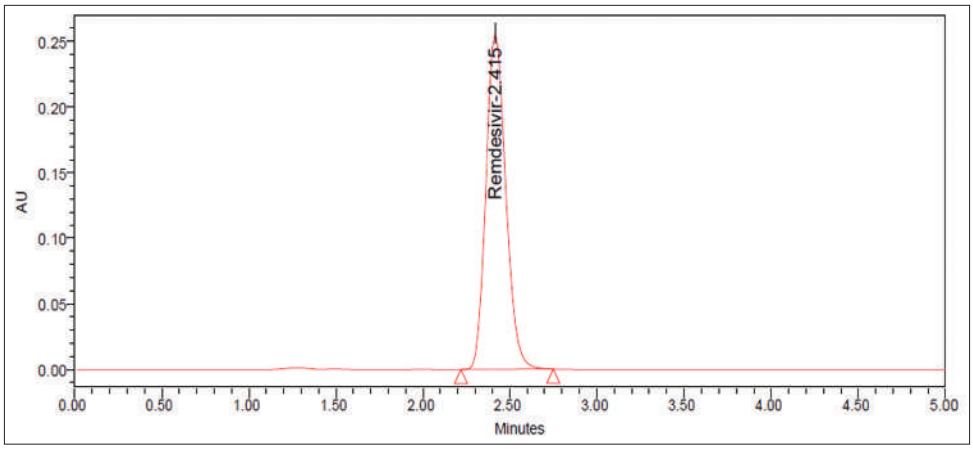


Figure 44: Interday precision chromatogram

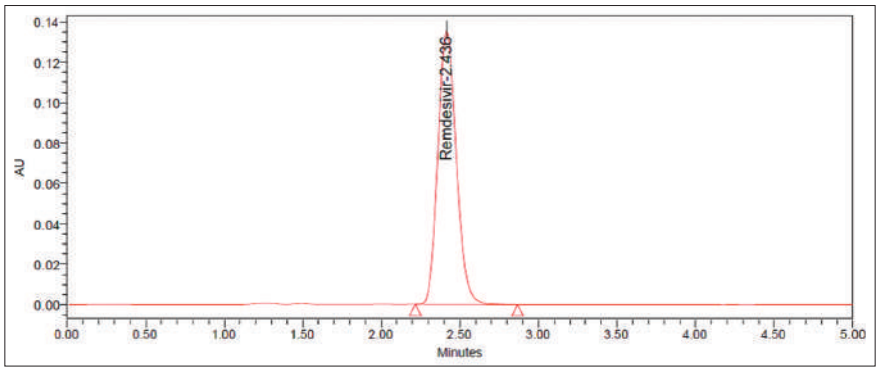


Figure 45: Chromatogram of accuracy 50%

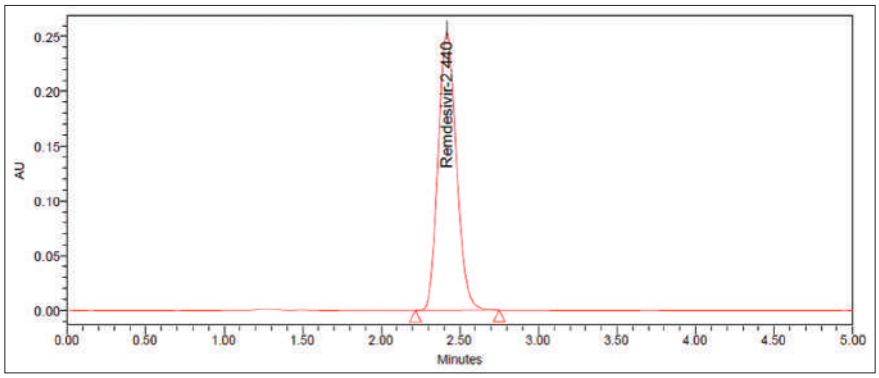


Figure 46: Chromatogram of accuracy 100%

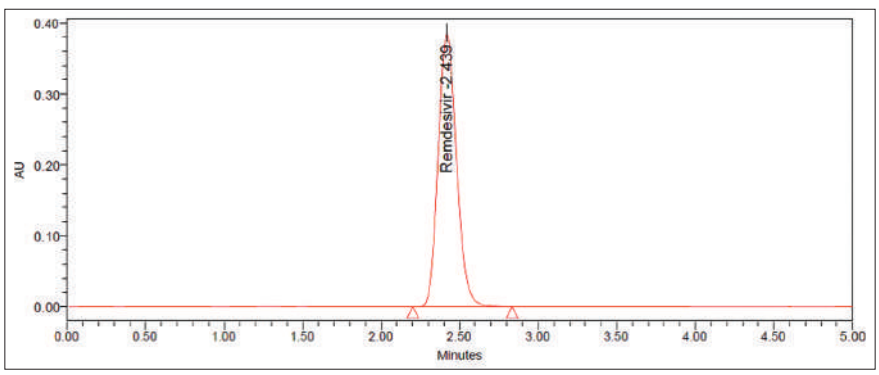


Figure 47: Chromatogram of accuracy 150%

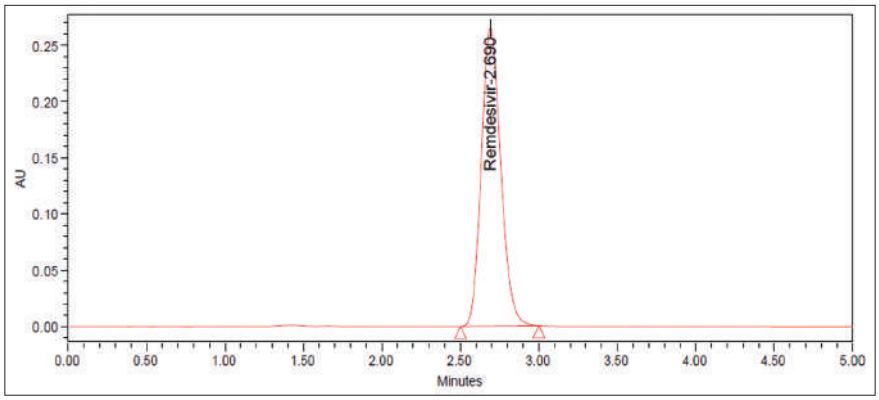


Figure 48: Chromatogram for less flow rate (0.8 ml)

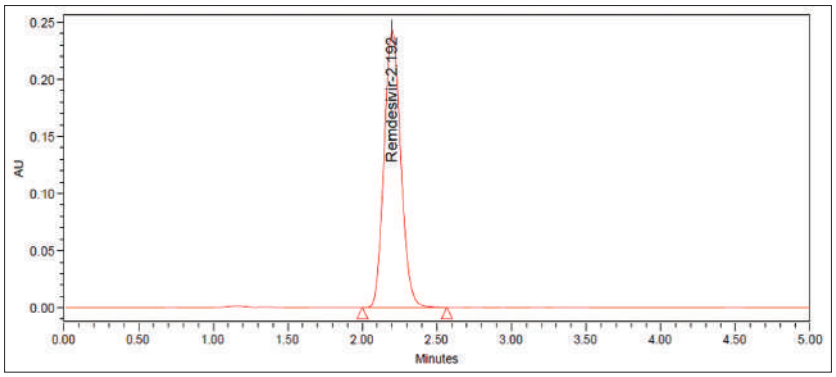


Figure 49: Chromatogram for more flow rate (1.2 mL)

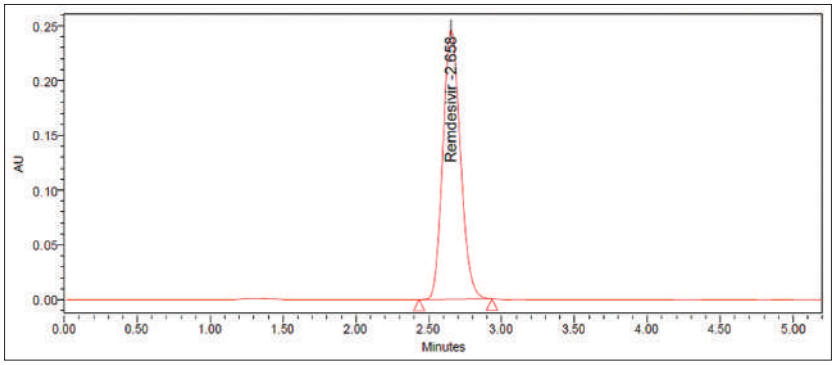


Figure 50: Chromatogram for less organic phase (63:37)

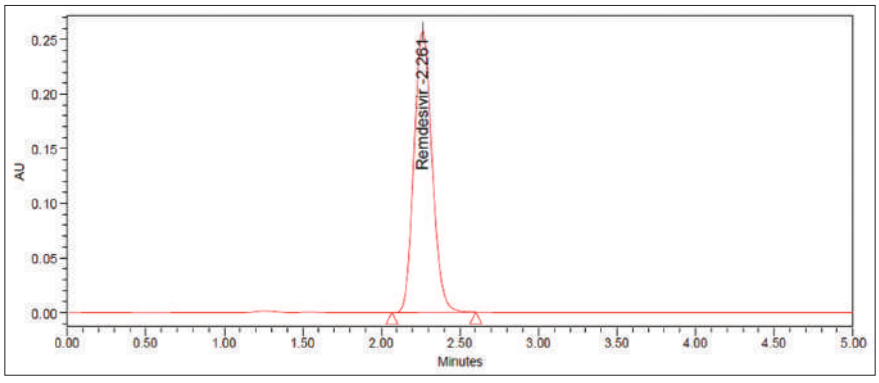


Figure 51: Chromatogram for more organic phase (77:23)

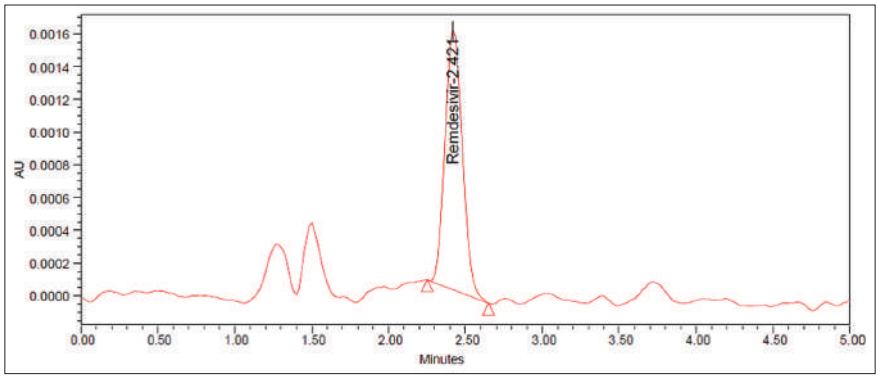


Figure 52: Chromatogram for LOD

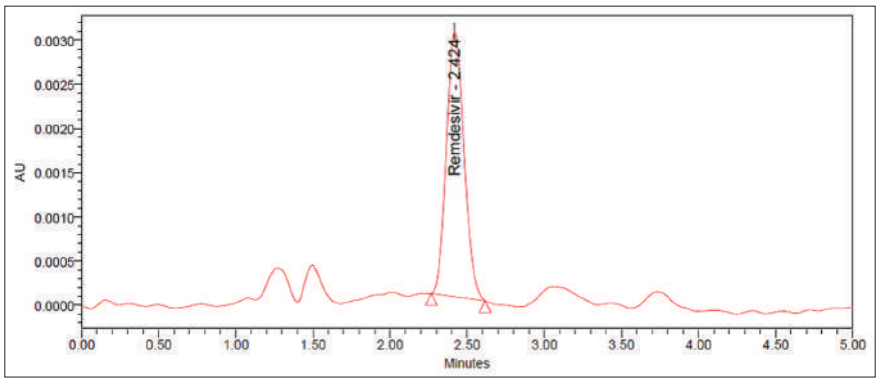


Figure 53: Chromatogram for LOQ

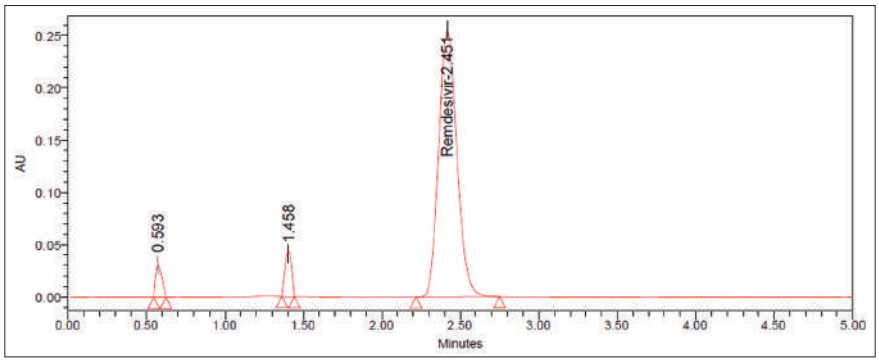


Figure 54: Chromatogram of acid degradation

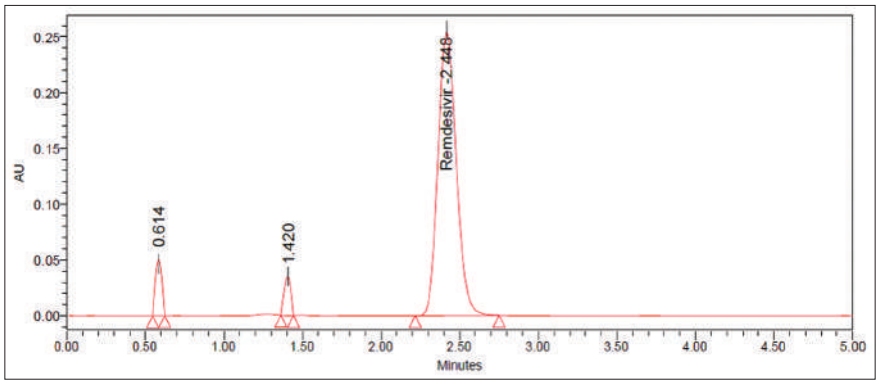


Figure 55: Chromatogram of alkali degradation

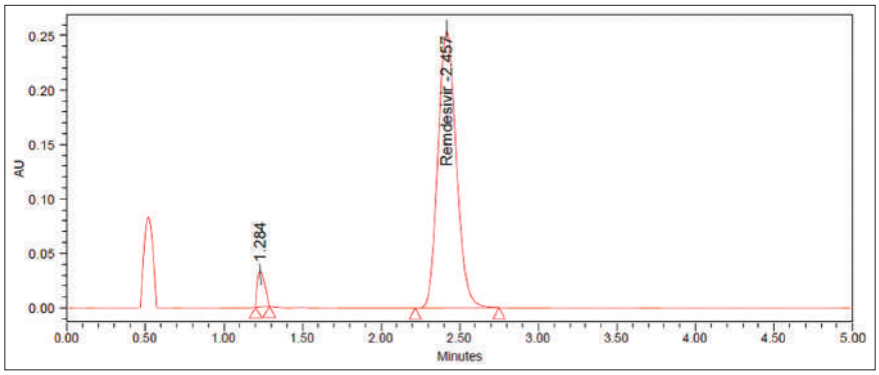


Figure 56: Chromatogram of peroxide degradation

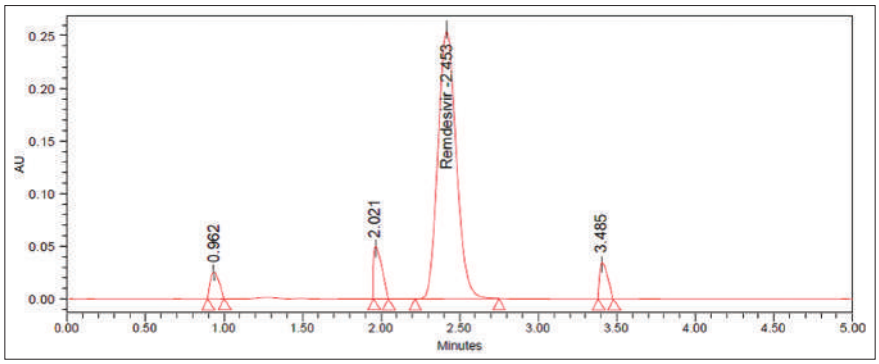


Figure 57: Chromatogram of reduction degradation

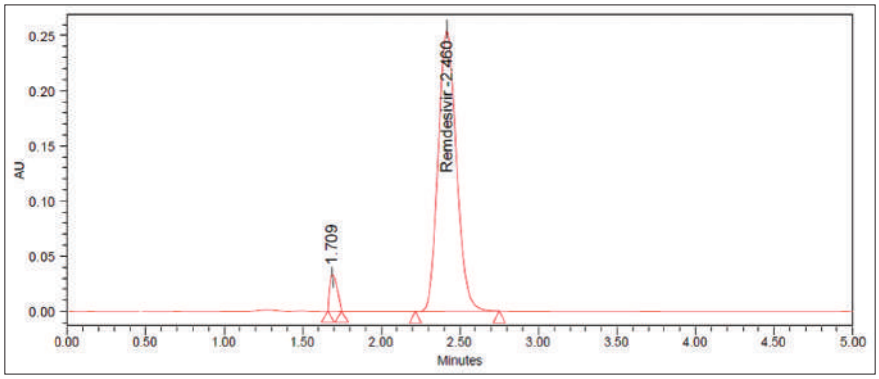


Figure 58: Chromatogram of thermal degradation

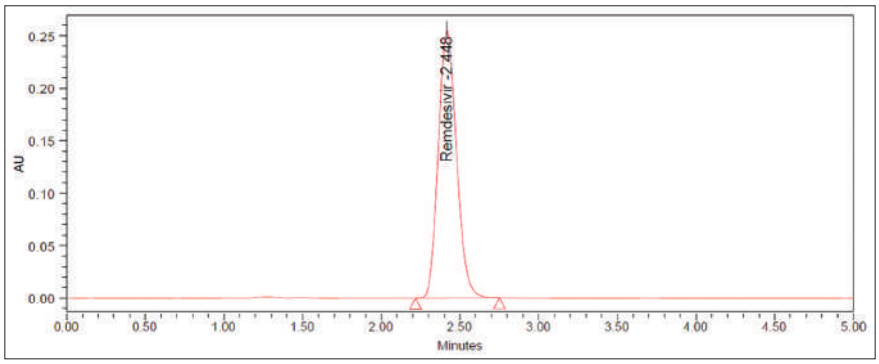


Figure 59: Chromatogram of photolytic degradation

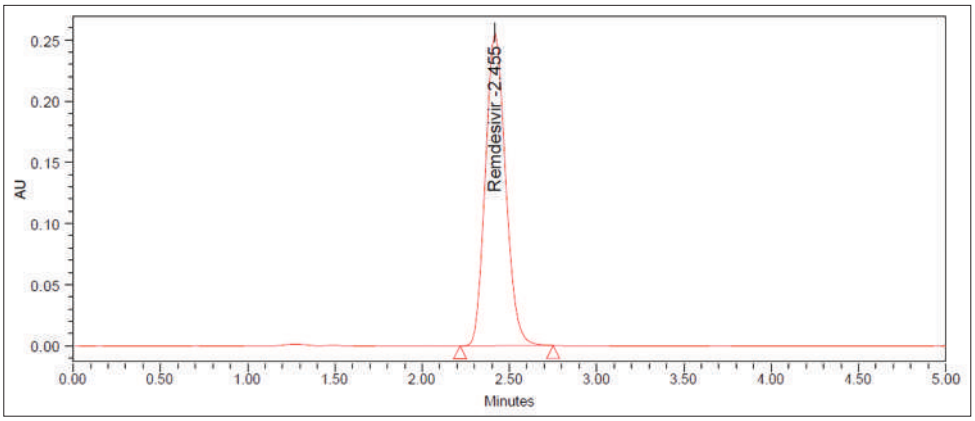


Figure 60: Chromatogram of control

102%). These indicate that the proposed method was accurate for the analysis.

CONCLUSION

The developed HPLC method for the estimation of selected drug is simple, rapid, accurate, precise, robust, and economical. The mobile phase and solvents are simple to prepare and economical, reliable, sensitive, and less time consuming. The sample recoveries were in good agreement with their respective label claims and they suggested noninterference of formulation excipients in the estimation and can be used in laboratories for the routine analysis of selected drugs. Since the system validation parameters of HPLC method used for estimation of selected drug in pure and have shown satisfactory, accurate and reproducible results (without any interference of excipients) as well, it is deduced that the simple and short proposed methods be most useful for analysis purpose. The present work concluded that stability indicating assay method by RP-HPLC was simple, accurate, precise, and specific and has no interference with the place and degradation products. Hence, these can be used for routine analysis of Remdesivir.

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