RP-HPLC Method for Development, Validation and Assay of Desidustat in Pharmaceutical Formulation

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Abstract— Desidustat is an orally bioavailable, hypoxia-inducible factor prolyl hydroxylase inhibitor and used to treat anemia of chronic kidney disease. An accurate, precise and robust reverse phase HPLC methods was developed and validated as per ICH guideline. An analytical column eclipse XDB C18 (150x4.6 mm,5µ) was used at flow rate 1.0 ml/min in the mobile phase ratio of 0.1% orthophosphoric acid and methanol 38:72 at 235 nm wavelength. These approach can be effectively utilized for the development and validation of desidustat in pharmaceutical formulation.

Index Terms- Desidustat, Eclipse XDB C18 (150x4.6 Mm,5µ), RP-HPLC, Development And Validation

I. INTRODUCTION

Chromatography is probably the most powerful and versatile analytical technique available to the modern chemist, its power arises from its capacity to determine quantitatively many individual components present in mixture in single analytical procedure. Its versatility comes from its capacity to handle a very wide variety of samples that may be gaseous, liquid or solid in nature. The sample can range in complexity from a single substance to multicomponent mixture containing widely differing chemical. The beginning of chromatography started with the work of botanist Michael Tswett in the year 1896. Tswett define chromatography as "The method in which the components of a mixture are separated on an adsorbent column in a flowing system". Recently, the IUPAC has defined chromatography as: “A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while other moves. The stationary phase may be a solid or a liquid supported on a solid or a gel may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid”.

1.11. High Performance Liquid Chromatography (HPLC) (44)

HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. Modern HPLC has many applications including separation, identification, purification and quantification of various compounds. Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical and biochemical research as well as for the pharmaceutical industry. These fields currently comprise only about 50% of HPLC users. Currently HPLC is used by a variety of fields including cosmetics, energy, food and environmental industries.

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography.

High pressure liquid chromatography was developed in the mid-1970's and has quickly improved with the development of column packing materials and the additional convenience of on-line detectors. New methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience

of HPLC. Improvements in type of columns and thus reproducibility were made as terms such as micro-column, affinity columns and fast HPLC began to emerge.

1.11.1. Advantages of HPLC

The major advantage of HPLC includes

1. It provides specific, sensitive and precise method for the analysis of different complicated samples.
2. There is ease of sample preparation and sample introduction.
3. There is increased speed of analysis.
4. It offers advantage over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermo-labile as well as non-volatile substances.

1.11.2 Most commonly used method in HPLC (45)

a) Normal phase chromatography

Normal phase HPLC was the first kind of HPLC chemistry used and separates based on polarity. This method uses a polar stationary phase and a non-polar mobile phase and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors and structural isomers. Use of more polar solvents in the mobile phase will decrease the retention time of the analyte while more hydrophobic solvents tend to increase retention time. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

Mechanism: Retention by interaction of the stationary phase polar surface with polarparts of the sample molecules.

1. Stationary phase: It is a bonded siloxane with polar functional group like SiO2,Al2O3, -NH2, -CN, -NO2, - Diol.
2. Mobile phase: Non-polar solvents like heptane, hexane, cyclohexane, chloroform,ethyl ether, dioxane.
3. Application: Separation of non-ionic, non-polar to medium polar substances.
4. Sample elution Order: Least polar components are eluted first.

B) Reverse phase chromatography

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe2SiCl, where R is a straight chain alkyl group such as C18H37 or C8H17.

The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. Reversed phase chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification.

RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte and the repulsive non-polar stationary

phase. The driving force in the binding of the analyte to the stationary phase is the decrease in the area of the non-polar segment of the analyte molecule exposed to the solvent.

Mechanism: Retention by interaction of the (stationary phase) non-polar hydrocarbonchain with non-polar parts of sample molecules.

1. Stationary phase: It is bonded siloxane with non-polar functional groups like n-octadecyl (C-18) or n-octyl (C-8), ethyl, phenyl, -(CH2)n-diol, -(CH2)n-CN.
2. Mobile Phase: Polar solvents like methanol, acetonitrile, water or buffer (sometimeswith additives of THF or dioxane).
3. Applications: Separation of non-ionic and ion forming non-polar to medium polarsubstances (carboxylic acids hydrocarbons).
4. Sample elution order: Most polar components are eluted first.

1.11.3. Components of the HPLC system

High performance liquid chromatography consists of following major components

a) HPLC gradient mixers
b) HPLC pumps
c) HPLC columns
d) HPLC detectors

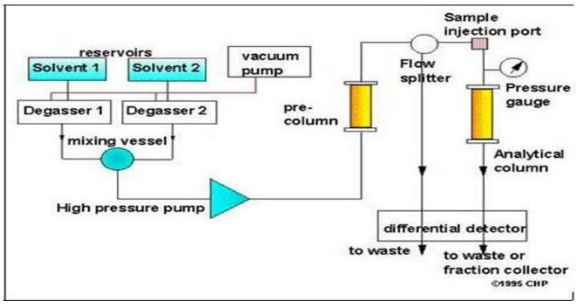


Fig. 7: Schematic diagram of HPLC instrument.

a) HPLC gradient mixers

HPLC gradient mixers provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes than when mixing large volumes. For low-pressure systems, it requires great precision in the operation of the miniature mixing valves used and low dispersion flows throughout the mixer. For multi-pump high-pressure systems, it requires a very precise control of the flow rate while making very small changes of the flow rate.

b) HPLC pumps

Because of the small particles used in modern HPLC column packing, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes, HPLC pumps should have flow rates that range from 0 to 10 ml/min, but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to a very constant flow rate at very low flow rate.

Pump Pressure

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 6000 lbf/inch2 (~40 MPa, or about 400 atmospheres). Modern HPLC systems have been improved to work at much higher pressures and therefore be able to use much smaller particle sizes in the columns (< 2 micrometers). These "Ultra High Performance Liquid Chromatography" systems or UHPLCs can work at upto 15,000 lbf/inch2 (-100 MPa or about 1000 atmospheres).

c) HPLC columns

Column is often referred to as the heart of the HPLC separation process. HPLC columns are packed with very fine particles (usually a few microns in diameter) to attain the low dispersion that give the high plate counts expected of modem HPLC. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase.

C18 and C8 HPLC columns

1. Classic reversed-phases for all general purpose applications.
2. Excellent peak shape and efficiency compared to competitive columns.
3. Classic reversed-phase retention and selectivity.
4. C18 is generally more retentive than the C8
5. Various factors that govern the retention of component are as follows:

Internal diameter

The internal diameter (i.d.) of an HPLC column is a critical aspect that determines of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of drug product for later use. Low i.d. columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

1. Larger i.d. columns (over 10 mm) are used to purify usable amount of materials because of their large loading capacity.
2. Analytical scale columns (4.6 mm) have been the most common type of column though smaller columns are rapidly gaining popularity. They are used in traditional quantitative analysis of samples and often use a UV-VIS absorbance detector.
3. Narrow-bore columns (1-2 mm) are used for applications when morr sensitivity is desired either with special UV-VIS detectors, fluorescence detectors or with other detection methods like liquid chromatography-mass spectrometry.
4. Capillary columns (under 0.3 mm) which are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5µm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter cubed. This means that changing to particles that are half as big in the same size of column will double the performance, but increase the required pressure by a factor of eight. Larger particles are more often used non-HPLC applications such as solid-phase extraction.

Pore size

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analyte. For example a protein which is only slightly smaller than a pore might enter the pore but not easily leave once inside.

d) HPLC detectors (46)

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. HPLC detectors use the same detection principles with extra care being given to the small solute elution volumes that result from the combination of high column efficiencies with small volumes. In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then peaks that appeared are with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volume decrease and system efficiencies increase the volume of the detector cell must also decrease. This is of course at odds for the requirement of detector to maintain high sensitivity, as this is usually dependant on having a larger cell volume. Again, this requires very careful design of modem detectors.

Types of detectors

1) Ultraviolet detector
a. Most widely used.
b. Principle-Absorption of UV visible light as the eluent from the column is passed through a small flow cell held in radiation beam.
c. Suitable for Gradient elution.
2) Fluorescence detector
a. Principle-Enable fluorescent compounds present in mobile phase to be detected by passing the column eluent through a cell irradiated with ultraviolet light and measuring any resultant fluorescent radiation.
b. Very sensitive and selective.
3) Refractive index detector

a. Principle-These are differential refractometer which respond to change in the bulk property of the refractive index of the solution of the component in the mobile solvent system.
b. It is less sensitive.

4) Electrochemical detector

a. Principle- These are based on standard electrochemical principles involving Zmperometry, voltametry and polarography.
b. These are very sensitive for substances that are electro active, i.e. those that undergo oxidation or reduction at a suitable potential.

5) Photodiode array detector (PDA)

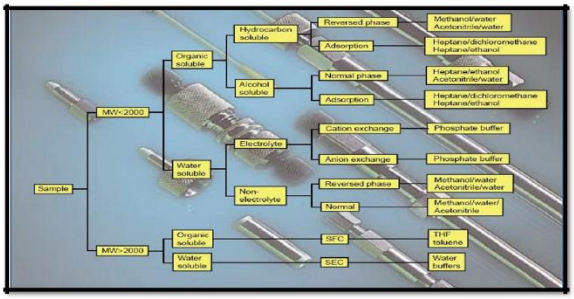
A photodiode array (PDA) is a linear array of discrete photodiodes on an integrated circuit (IC) chip. For spectroscopy it is placed at the image plane of a spectrometer to allow a range of wavelengths to be detected simultaneously. In this regard it can be thought of as an electronic version of photographic film. Array detectors are especially useful for recording the full UV-visible absorption spectra of samples that are rapidly passing through a sample flow cell, such as in an HPLC detector.

1.11.4 Method development in HPLC

Methods for analyzing drugs by HPLC can be developed, provided one has knowledge about the nature of the sample namely, its molecular weight, polarity, ionic character, pKa values and the solubility parameter. An exact recipe for HPLC method development cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.

The water soluble active phamaceutical ingredients is further differentiated as ionic or non ionic which can be separated by reverse-phase. Similarly, the organic soluble API can be classed as polar and non-polar and equally separated by reverse phase. In some cases the non-polar API may have to be separated using adsorption or normal phase HPLC, in which mobile phase would be non-polar organic solvent.

Fig. 8: General considerations for selection of mobile phase



Role of different parameters for selection of mobile phase

a) pH

pH is a critical factor which affects the selectivity of the separation process in reversed phase HPLC. It is necessary to select the proper buffer pH to reproducibly separate ionizable compounds by reversed-phase HPLC. Selecting an improper pH forionizable analyte often leads to asymmetric peaks that are broad with tailing, splitting or shouldering of peak. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviation (RSD) between injections and reproducible retention time. Sample retention increases when the analyte is more hydrophobic. Thus when an acid (HA) or base (BOH) is ionized (converted from the unionized free acid or base) it becomes more hydrophilic (less hydrophobic, more soluble in aqueous phase) and less interacting with column binding sites, as a result the ionized analyte is less retained on the column. When the pH = pKa for the analyte, it is half ionized, i.e. the concentration of the ionized and unionized species are equal.

b) Buffer

In reverse-phase liquid chromatography, the mobile phase pH values are usually between 2.0 and 7.5. Buffers are needed when an analyte is ionizable under reverse-phase conditions or the sample solution is outside this pH range. Analyte ionizable under reverse-phase conditions often have amine or acid functional groups with pKa between 1.0 and 11.0. A correctly chosen buffer pH will ensure that the ionizable functional group is in a single form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring

the pH of the injected solution to a less harmful pH. In general, potassium phosphate buffer at pH 3.0 is an excellent buffer for analyte that contain acid and amine functional groups. The potassium salt works better than the sodium salt for amines.

c) Temperature
Although the effect of temperature is less significant in liquid chromatography, it may play a significant role in the optimization for difficult separations. Temperature variations over the course of a day have quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While temperature is a variable that can affect the selectivity, its effect is relatively small. Also retention time generally decreases with a increase in temperature for neutral compounds but less dramatically for partially ionized analyte. Snyder reported that an increase of 1˚C will decrease the retention time by 1 to 2%.

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 slower 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.

e) Selection of detector
Detector is the eye of the HPLC system and measures the compounds after their separation on the column. There are basically two types of detectors-. the Bulk property detectors and Solute property detectors. Detectors, in order of their popularity are UV, fluorescent, conductivity and refractive index detectors. UV detector is the first choice because of its convenience and applicability in case of most of the samples.

The latest versions of HPLC equipments are available with Photo Diode Array detectors. The response obtained from a given detector will vary according to the nature of solute molecule.

f) Sample preparation
The sample prepared should be homogeneous. It should be completely soluble in the selected solvent; usually the solvent used to dissolve the sample should be the mobile phase itself or any solvent miscible with the mobile phase.

g) Chromatographic separation
After achieving a resolution with a pre-optimized solvent system, to obtain reproducible results following criteria must be satisfied-
1. Monitoring flow rate.
2. Keeping the solvent composition intact.
3. Solvent system must be covered before storage.
4. Monitoring column temperature.

1.11.6. System suitability parameters (48)
a) Theoretical plates (N)
It is also called as column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid-liquid or so1id-liquid phase occurs. The number of theoretical plates in column is given by the following relationship

$N = 16 (tR/w) 2$
Where,
tR is the retention time and w is the width at the base of the peak.
$HETP = L/N$

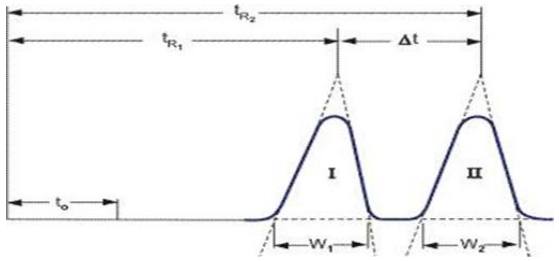
Where,
L is the length of column.
Theoretical plates should be more than 2000.

b) Height equivalent to theoretical plate (h)
The efficiency of the column can be expressed as the height equivalent to theoretical plates (HETP). Lower the HETP, higher is the efficiency of the column i.e. higher the theoretical plate, more efficient is the column.
$h = L / N$

Where,
h = height equivalent to theoretical plate (HETP),
L = Length of the column,
N = Number of theoretical plates.

c) Retention time (tR)
It is the time between which the sample is injected and the chromatographic peak is recorded.

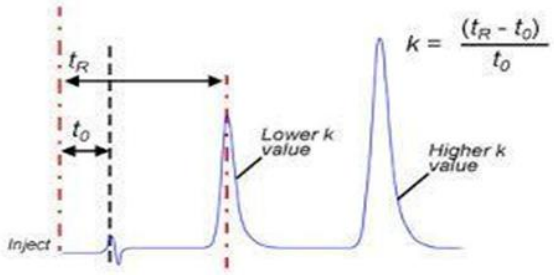
d) Resolution (Rs)
It is a measure of quality of separation of adjacent bands in a chromatogram; bands have small Rs values. It is calculated from the width and obviously overlapping retention time of two adjacent peaks.



Rs=2(t2 -t1)
W1/ W2
Where,
t1 and t2 are the retention time of first and second adjacent bands and
W 1 and W2 are their baseline bandwidths.
Reliability of calculation is poor if Rs is < 1.0.

e) Capacity factor (k')
It is the measure of the position of a sample peak in the chromatogram, being specific for a given compound. k' depends on the stationary phase, mobile phase, p temperature and quality of column packing.

For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10.



k'1=t R1t-t 0
k'2=tR2t-0t0

Where,
tR1 and tR2 are total retention time of component 1 and 2 respectively.
t0 is non-adsorbed time.

f) Separation factor (Selectivity) (α)
It is a measure of peak spacing and is expressed as:
k 2 '=t R2 /t 0
k1 = t R1/ t 0

g) Tailing factor (T)
It is the measure of peak symmetry and is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$T \quad \frac{W0.05}{2F}$$

Where,
W0.05 is the width of peak at 5% height and
F is the distance from the peak maximum to the leading edge of the peak
height from the baseline.

1.11.7. Validation of analytical techniques (49)
Introduction to validation
Validation is a concept that has been evolving continuously since its first formal appearance in United States in 1978. The concept of validation has expanded through the years to encompass a wide range of activities from analytical methods used for the, quality control of drug substances and products to computerized system for clinical triallabeling or process control. Validation is the overall expression for a sequence of activities in order to demonstrate and document that a specific product can be reliably manufactured by the designed processes, usually, depending on the complexity of today's pharmaceutical products, the manufacturer must ensure; "that products will be consistently of a quality appropriate to their intended use".

Validation is a proof that a process works and this must be done using scientific ad statistical principles. This is done to establish process capability and to confirm end acceptability. Validation determines process variables and the acceptable limits for these variables and accordingly sets up appropriate in process controls, which specifies alert and action levels.

ICH guideline Q2 (R1) Method Validation Parameters are as follows:-
a) Accuracy
b) Precision
c) Specificity

d) Linearity
e) System suitability
f) Ruggedness
g) Robustness

a) 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. This is sometimes termed trueness.

It is measured as the % of analyte recovered by assay or by spiking samples in a blind study. Accuracy should be established across the specified range (that is, line of working range) of the analytical procedure. For the assay of the drug substance, accuracy measurements are made by comparison of the results with the analysis of a standard reference material or to compare the results obtained from a second well-characterized independent procedure, the accuracy of which is stated and/or defined.

ICH Guidelines Q2(R1) recommend assessment of accuracy at three levels covering the specified range (i.e. three concentration levels and three replicates at each level of the total analytical procedure). The data should be reported as the percent recovery of the known amount added or as the difference between the mean and true values with confidence intervals.

The % recovery was then calculated by using formula.

$$\% \text{ Recovery} = \frac{(A - B) \times 100}{C}$$

Where,
A: % Total amount of drug estimated
B: % Amount of drug found on preanalyzed basis
C: % Amount of pure drug added.

b) 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 samples. It is expressed as standard deviation or coefficient of variation.

Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability: Repeatability expresses the precision under the same operating conditionsover a short interval of time. It is also termed as intra-assay precision.

Intermediate precision: Intermediate precision expresses within-laboratories variations:different days, different analysts, different equipment, etc.
Reproducibility: Reproducibility expresses the precision between laboratories(collaborative estudies, usually applied to standardization of methodology).

c) Specificity
It is the ability to assess unequivocally the analyte in the presence of components which may he expected to be present in the sample under consideration. This might include degradants, impurities, matrices, excipients etc.

This definition has the following implications:
(i) Identification: to ensure the identity of an analyte.
(ii) Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.
(iii) Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

e) Linearity
The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration

or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods.

f) System suitability

System suitability is a Pharmacopoeial requirement and is used to verify whether the resolution and reproducibility of the chromatographic system is adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard solutions.

g) Ruggedness

Ruggedness is the degree of reproducibility of the results obtained under a variety expressed as % RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under variety conditions such as different laboratories and different days etc.

h) Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Perform experiments by changing conditions such as temperature (±5 °C), change in wavelength (±2 nm), ionic strength of buffers and level of additives to mobile phase.

Table No. 8: Validation Parameter([50])

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES Quantition Limit		ASSAY-dissolution (measurement only) - content /potency
Accuracy	-	+	-	+
Precision	-	+		+
Repeatability	-	+	-	+ (1)
Intermediate Precision	+	+ (1)	-	+
Specificity (2)	-	+	+	-
Detection Limit	-	- (3)	+	-
Quantition Limit	-	+	-	+
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated
+ Signifies that this characteristic is normally evaluated

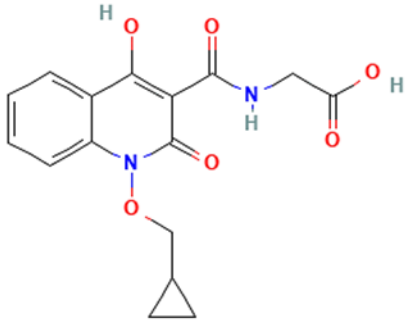
(1) In cases where reproducibility has been performed, intermediate precision is not needed.

(2) Lack of specificity of one analysis procedure could be compensated by other supporting analytical procedure(s).

(3) May be needed in some cases.

DRUG PROFILE OF DESIDUSTAT [2]

- Structure – Desidustat



IUPAC Name - 2-[[1-(cyclopropyl methoxy)-4-hydroxy-2-oxoquinoline-3-carbonyl] amino] acetic acid.

- Molecular formula - C16H16N2O6
- Molar mass - 332.31 mol -1
- Density - 1.5±0.1 g/cm3
- Solubility-DMF: 3mg/mL
  - DMSO: 3mg/mL
  - Ethanol: 1mg/mL
  - Methanol: 1mg/ml
- Uses - Treatment of anemia of chronic kidney disease.
- Dose: - 50 mg

Table No 8: Details of dosage form of Desidustat

Brand Name	Composition	Company	Packing
OXEMIA	Desidustat 50mg	Zydus pharmaceutical	6

VII. EXPERIMENTALWORK

7.1. Chemicals and Reagents

Pure drug sample of Desidustat was generously acquired as gift sample from Zydus Pharmaceuticals Pvt. Ltd. (Gujrat, India). HPLC grade acetonitrile, methanol and water were used. All the solvents and mobile phase were filtered through Nylon filter (0.45µ) and ultrasonicated before use. All the sample solutions for HPLC study were filtered through Nylon filter (0.45µ) before use. Other reagent hydrogen peroxide, hydrochloric acid, sodium hydroxide, ortho phosphoric acid used were of AR grade.

Tablet formulation OEXMIA containing Desidustat (50mg) was purchased from the Local Pharmacy manufactured by Zydus Pharmaceuticals Pvt. Ltd.

Table No 9: -List of grade Solvent

Sr.No.	Reagent	Grade	Manufacturer
1	Water	HPLC Grade	MERCK
2	Acetonitrile	HPLC Grade	MERCK
3	Methanol	HPLC Grade	MERCK
4	Acetic acid	HPLC Grade	MERCK
5	Ortho phosphoric acid	------	-------

7.2. Instruments

1. Shimadzu UV 1800 double beam UV-visible spectrophotometer was used along with1.0cmpath length matched pair of quartz cell.
2. Agilent- 1260 HPLC system comprising of Quaternary gradient pumps G1311A withonlineDegasserG1322A,Variable wavelength UV-VIS detector G1314B,Manual Rheodyne injector 7725 I with 20 ul loop and Kromasil- 5C-18 column (150mm, 4.6 mmi.d., 5um).
3. Digital Balance (ShimadzuAUY-220, Japan)
4. Ultra-Sonicator (PCI services, Mumbai).
5. Digital pH meter (Elico LI-120).
6. Hot air oven (Meta-Lab Scientific industries, Mumbai)
7. Digital water bath (Yorco Scientific Industries Pvt Ltd.).

7.3. Determination of wavelength for detection of Desidustat

The working standard solution of Desidustat (10 ug /ml in methanol) was scanned in the range of 200-400 nm against solvent blank and spectrum was recorded. UV spectrum shows λmax at 235 nm, was selected as wavelength of detection for HPLC study. The UVspectrum of Desidustat is depicted in figure7.

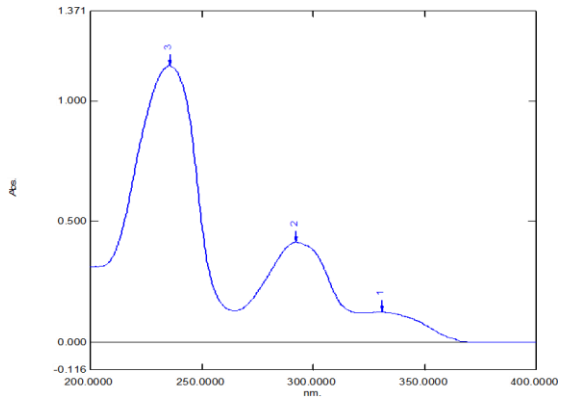


Fig7: -UV Spectrum of Desidustat

7.4. Preparation of Standard Solutions

7.4.1. Stock standard solution of Desidustat

Accurately weighed 10.0 mg of Desidustat was dissolved and diluted to methanol in a 10mlvolumetric flask (conc. 1mg/ml).

7.4.2. Working standard solution of Desidustat

One ml from above stock solution was diluted with mobile phase in a 10ml volumetric flask (conc. 100ug/ml). One ml from this solution was further diluted to 10ml with mobile phase to give (10 µg/ml) concentrations.

7.4.3. Preparation of sample solution

Accurately weighed quantity of tablet powder equivalent to 10mg (24.6 mg) Desidustat was transferred to10mlvolumetric flask and dissolved in about 7mlmethanol using ultrasonication for 10min. The volume was made upto the mark with methanol.

The solution was filtered through Nylon filter (0.45µ) and One ml of filtrate was diluted to10ml with mobile phase to obtain concentration 100 µg/ml. One ml from this solution was further diluted to 10 ml with mobile phase to obtain a concentration of10ug /ml.

7.5. Optimization of Chromatographic Conditions

For the optimization of chromatographic conditions several trials were taken, some of the trials and their chromatograms are depicted.

7.6. Chromatographic conditions

Different solvent and buffers of different pH were tried by permutation and combination to obtain adequate retention of the drug. Finally, mixture of Methanol: 0.1 %Ortho phosphoric acid in the ratio of72:28 % v/v was found to yield satisfactory retention time of desidustat at 4.7 min, with sharp symmetrical peak and well resolved from all the degradation products. One of the chromatograms of standard solution of Desidustat are depicted in Figure No: -12

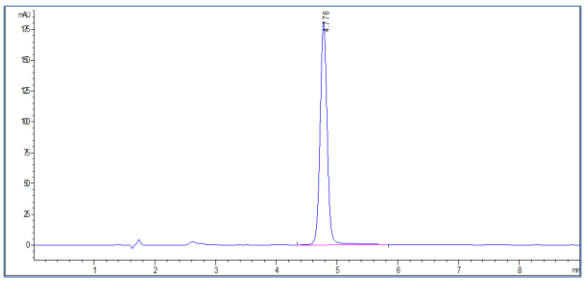


Fig.12:-HPLC chromatogram of standard desidustat in Methanol: 0.1% Ortho phosphoric acid (72:28 %v/v)

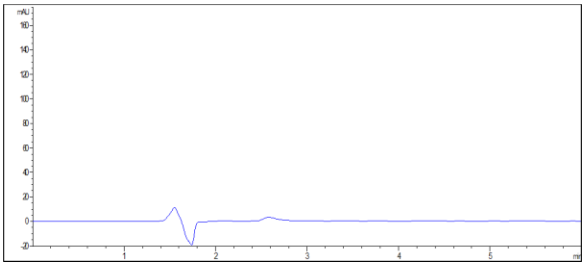


Fig.13:-HPLC chromatogram of Blank Methanol: 0.1% Ortho phosphoric acid (72:28 %v/v)

The following chromatographic condition were maintained throughout the method development

Column	Eclipse XDB C18 column [(150x4.6) mm i.d, 5µm]
Mobile phase	Methanol :0.1% ortho phosphoric acid (72:28)
Flow rate	1 ml/min
Column Temp	25℃
Sample size	10 µl
Detection wavelength	235 nm
Injection volume	20 µl

Development & validation of RP-HPLC for the estimation of Desidustat in bulk drug & Pharmaceutical formulation.

In the RP- HPLC method, the chemical and reagents like methanol, acetonitrile, water, & ortho phosphoric acid used were of HPLC grade throughout the experimentation. All the solvents and mobile phase were filtered through Nylon Filter (0.45µ) & ultrasonicated before the use. All the sample solutions for HPLC study were filtered through Nylon filters (0.45µ) before use. The other reagents such as H2O2, NaOH, and HCl used were of AR grade.

The HPLC Agilent 1260 infinity II, system comprising of Quaternary gradient pumps with UV detector & Eclipse XDB column (150mm×4.6mm, i.d.5µm) was used throughout the study.

8.1.1. Preparation of a standard Desidustat solution.

10 mg of Desidustat was dissolved to 10ml of methanol and diluted further appropriately with mobile phase to give 10 µg /ml conc.

8.2. Determination of wavelength for detection of Desidustat

In this the working standard solution of desidustat 10ug/ml in mobile phase) was scanned in the range 200-400 nm against the solvent blank in the diode array detector & spectrum was recorded. The UV spectrum shows λmax at 235 nm was selected for the wavelength of detection for HPLC study. The UV spectrum of desidustat is depicted in figure no. 7.

8.3. Optimization of chromatographic condition.

For the optimization of chromatographic conditions, several trials by using permutation and combination of

different solvents and 0.1% Ortho phosphoric acid were used to get adequate retention time of drug with sharp and symmetrical peak & adequate theoretical plate, finally a mixture of methanol and 0.1% ortho phosphoric acid in the ratio 72:28 % v/v was found to yield satisfactory retention time of desidustat 4.71 min with sharp symmetrical peak and we'll resolved from all degradation products, the HPLC chromatogram of standard solution desidustat was depicted in fig. no - 12

8.2.2.3 Assay:

Percentage purity of Desidustat from its tablet formulation was determined by proposed method. Average percentage purity for six replicates of test concentration was found to be 98.71%. The method was found suitable for the assay of Desidustat with % RSD 0.703 less than 2 %.

Table No 33: - Summary result of Assay

Statistical parameter	% Desidustat Content
Mean	98.71
±SD	0.694
%RSD	0.703

Validation of method

Developed chromatographic method was validated as per ICH guidelines for specificity, precision, accuracy, linearity, ruggedness and sensitivity.

a) System suitability test parameters

For system suitability test parameters, six replicate injections of working standard solution of Desidustat (10ug/ml) each were injected and analysed under optimized chromatographic conditions. The results of system suitability test parameters study are depicted in Table No. 34

Table No 34: Summary of result of System Suitability

Statistical parameter	Retention time (min)	Capacity factor (k')	Peak area	Symmetry	Plates
Mean	4.75	2.18	1465.48	0.94	8475.28
SD	0.033	0.019	25.083	0.017	168.571
%RSD	0.695	0.895	1.711	1.883	1.989

b) Linearity Study:

Linearity study was carried out over concentration range 5-25 µg/mL. Stock solution was prepared in methanol and working solution in mobile phase. Each concentration was injected and peak area considered in linearity plot.

Results of linearity study show that linear response as function of concentration. Regression coefficient was found to be 0.998. Slope and intercept equation was found to be y = 186.82x-223.5 Slope and intercept equation was used for estimation of percentage content of Desidustat from its tablet dosage form.

Table No 35: - Summary of result of linearity study

Concentration Range	5-25µg/ml
Equation for straight line	186.82x-223.5
Correlation Coefficient	0.998
Slope	186.82x

c) Method Precision:

Precision of method was confirmed from results of repeatability study performed on same day for six replicates of 100 % test concentration. % RSD of Intraday study was found 0.432 less than 2%, ascertained repeatability of method.

Table No 36: - summary of precision result

Statistical parameter	% drug content
Mean	100.11
std	0.432
%RSD	0.432

d) Specificity

These studies were carried out to ascertain how accurately and specifically, the analytes of interest are estimated in presence of other component (e.g., impurities, degradation product) by exposing the tablet powder sample to different stress condition such as acid, alkali, oxidation, heat and light and then analyzing them by proposed method (table no: - 31).

The result of estimation of desidustat tablet under different stress condition was quite concurrent with that of standard (API). The well resolved degradants peak was also observed in the respective chromatogram. The result were unaltered in case of photolysis, thermal, basic hydrolysis, oxidation stress indicating no degradation of drug and have not show any evidence of additional peak. This indicates that the method is selective and capable of estimating intact drug content free of interference from its degradation product. Further, the method may be made capable of estimating various degradant if appropriate standard of individual degradation product are generated.

e) Accuracy:

In accuracy study, % recovery was measured at 80%, 100% & 120% of test concentrations. Standard solutions of desidustat 3mg, 5mg, 7mg was added to test concentration of sample. % recovery of added concentration was determined in triplicates. Average % recovery was found to be in range 98-100 %. Statistical values of recovery study showed in table-24 indicating that developed method was accurate

Table No 37: - summary of accuracy study of proposed HPLC method

Recovery Level (%)	Statistical values of % recovery		
	Average	SD	%RSD
80%	100.03	0.057	0.57
100%	99.33	1.157	1.162
120%	100.33	0.577	0.575

f) Robustness:

Robustness study was carried out with deliberate change in three parameters i.e., detection wavelength, flow rate, mobile phase. Detection wavelength was changed by ±2 nm on either side of initial value while flow rate changed by± 0.1 mL/min on either side of initial flow rate and mobile phase change by ±2 v/v on either side of initial mobile phase. Peak area and retention time was found to be changed with robustness parameters i.e., increased at lower values of robustness parameters and vice versa. No significant change was observed in % content of desidustat. Data of robustness study was reported in Stress conditions. Statistical value of robustness study was shown in table -38.

Developed method was found to be robust as % RSD found less than 2%.

Table No 38: - Summary of Robustness result.

Sr. No	Parameter	Optimized condition	Used condition	Peak condition	Retention time (t_R)	Plate count	Peak symmetry
1	Flow rate (±0.1 ml/min)	1ml/min	0.9 ml/min	1430.20	5.3	8646	0.94
			1.1 ml/min	1409.63	4.4	8495	0.92
2	Detection wavelength (±2nm)	235 nm	233 nm	1563.62	4.7	87199	0.93
			237 nm	1496.80	4.7	87177	0.92
3	Mobile phase (±2v/v)	72:28v/v	70:30v/v	1495.44	5.4	87944	0.94
			74:26v/v	1291.50	4.0	86499	0.92

g) Ruggedness

Ruggedness of the proposed method was ascertained by analyzing sample on same day at three different intervals, on different days and by three different analysts using similar operational and environmental conditions. The result of estimation of sample on intra-day, inter day and by different analyst were very much reproducible with maximum % RSD of the order of 0.58 (Table No.26). This indicates the ruggedness of the method in the hands of different expert analyst and

at different time intervals. The results are shown in table no.39.

Table No 39: - Summary of Result of Ruggedness study

Statistical parameters	% estimation (n=3)		
	Intra-Day	Inter-day	Different Analyst
Mean	99.56	98.93	98.93
± SD	0.950	0.577	0.577
% RSD	0.954	0.583	0.583

h) LOD and LOQ

LOD and LOQ for desidustat were evaluated by injecting a series of solutions duly diluted with known concentrations. Based on the response and slope of regression equation of the parameters of LOD and LOQ were calculated by using formula.

The LOD of drug was found to be 1.345 µg/ml
The LOQ of drug was found to be 4.078 µg/ml

CONCLUSION

In the present project work, a stability indicating RP-HPLC method was developed and validated for the estimation of Desidustat in tablet formulation. The force degradation study carried out on drug showed significant degradation product generated under the various condition of exposure. All the degradation products are well resolved from parent drug under the optimized chromatographic condition.

Moreover, the method is in true sense can be said to be specific stability indicating assay method for Desidustat due to its estimation the drug content unequivocally free of interference from its degradation product.

The validation of method indicates that method is simple, precise, accurate, rugged and specific for the estimation of both in pharmaceutical formulation.

The proposed RP- HPLC method can be adopted for estimation of Desidustat and fixed dose formulation estimation of Desidustat in routing quality control in the pharmaceutical industries.

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