ORIGINAL ARTICLE

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J. Chem. Metrol. 17:2 (2023) 215-224

journal of chemical metrology

Reversed phase-HPLC-PDA method for quantification of Desidustat

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(Received July 18, 2023; Revised November 01, 2023; Accepted November 02, 2023)

Abstract: The hypoxia inducible factor prolyl hydroxylase inhibitor Desidustat is used to treat anemia linked to chronic kidney disease (CKD). For the estimation of Desidustat in bulk and commercial tablet formulation known as Oxemia, a precise, accurate, and sensitive reverse phase HPLC method has been developed and validated. The method described here was optimised with a Hypersil C18 (250 × 4.6 mm, 5 µm) column serving as the stationary phase and a mobile phase that included methanol: acetonitrile (80:20 v/v) added to the column at a flow rate of 1 mL/min. Using a photo diode array detector, Desidustat was detected at an analytical wavelength of 230 nm. With a correlation coefficient of 0.9989, the developed method was found to be linear in the concentration range of 1–6 µg/mL. Every parameter listed in the in International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Quality 2 (Revision 1) guideline was verified for the described method.

Keywords: Desidustat; HPLC; method validation.

1. Introduction

The chemical name for Desidustat (DES) is 2-{[1-(cyclopropyl methoxy)-4-hydroxy-2- oxo-1, 2-dihydroquinolin-3- yl] form amido} acetic acid [1] and it's structure is depicted in Figure S1 in supporting informaiton. It is an agent in the category of antianemia. Desidustat has a molecular weight of 332.31 g/mole and the formula $C_{16}H_{16}N_2O_6$ [2]. Desidustat's log P value is 0.57 and its pKa value is 3.17. Desidustat is a tiny molecule that inhibits the hydroxylase and promotes erythropoiesis in response to hypoxia inducible factor. As a result, it is used to treat anemia brought on by chemotherapy, chronic kidney disease, and other conditions. The only published articles on the subject have been clinical in nature. Recently, two stability-indicating RP HPLC methods and one planar chromatographic analytical method have been reported for the estimation of Desidustat. These reports have led to the development of a simple reverse phase high performance liquid chromatographic method to determine Desidustat at low concentration levels [3–15]. Drugs are retained on liquid-coated solid supports (columns) when they are dragged through the column with mobile phase in high performance liquid chromatography, which is renowned for its accurate, precise, and dependable results. Polar mobile phase and non-polar stationary phase are indicated by the reverse phase. Reverse phase chromatography is a reliable, accurate, and precise method for quantifying newer molecules, such as Desidustat, both in bulk and in pharmaceutical tablet dosage forms, such as Oxemia tablets.

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The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology July-December 2023 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.95.2307.2839
Available online: November 09, 2023

2. Experimental

2.1. Standard Drug, Sample (Formulation) and Chemicals

Desidustat (purity 99.34%) was a gift from India's reputable pharmaceutical industry. I bought an Oxemia Tablet strip (Zydus Pharmaceutical Ltd.) containing 25 mg of Desidustat from the local market. The source of Whatman filter paper 42 was Merck KGA in Germany. Solvents, HPLC-grade methanol, and HPLC-grade acetonitrile acid were procured from SRL Chemicals Pvt. Ltd. located in Ahmedabad, Gujarat, India.

2.2. Analytical Wavelength Selection

A concentration of 10 µg/mL solution of Desidustat was produced using methanol and scanned with a double beam UV-visible spectrophotometer in the 400–200 nm wavelength range. The drug was found to absorb significantly at 230 nm, which was chosen as the analytical wavelength.

2.3. Chromatographic System

The method was developed using an HPLC equipment consisting of a waters system with a column Hypersil C18 (250 × 4.6 mm, 5 µm), Empower software, and a photo diode array detector system. A stationary phase of Hypersil C18 (250 × 4.6 mm, 5 µm) was used, and a mobile phase of methanol: acetonitrile (80:20 v/v) with pH 3.0 adjusted with orthophosphoric acid was chosen. Ten milliliters of Desidustat were added to the system along with a steady flow rate of 1 mL/min for the mobile phase. The detection process was run at a chosen analytical wavelength.

2.4. Standard Solution Preparation

10 mg of Desidustat was accurately weighed and then added to a 10 mL volumetric flask. A small amount of methanol was then added to the flask to dissolve the contents, and the volume was adjusted with methanol to produce a solution with a concentration of 1000 µg/mL. Subsequent dilutions were performed until the solution contained 100 µg/mL of Desidustat. A working standard concentration of 10 µg/mL was prepared using the proper dilution.

2.5. Calibration Curve Determination

1 mL, 2 mL, 3 mL, 4 mL, 5 mL, and 6 mL were taken out of the 10 µg/mL stock solution and put into various volumetric flasks. The volume was then adjusted using mobile phase to get the concentration range of 1 to 6 µg/mL. An injection volume of 10 µL was added to the RP-HPLC apparatus for every concentration, and a calibration curve was produced.

2.6. Validation

Validation of developed RP-HPLC method was carried out as per International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2 (R1) [16].

2.6.1. Linearity

Five determinations of the chromatogram were made to test the linearity of the developed method for Desidustat estimation over a concentration range of 1 to 6 µg/mL. A graph of peak area versus concentration was plotted to establish the calibration curve, and a straight-line equation was obtained.

2.6.2. Precision

The degree of agreement between several measurements made from repeated samplings of the

same homogeneous sample under specified circumstances is expressed as precision. A repeatability study concerning injection repeatability was conducted. To achieve injection repeatability, the middle concentration (3 µg/mL) of Desidustat from linearity range was injected. The same concentration was injected six times to test for repeatability.

Two distinct studies, one intraday precision and the other interday precision, were used to conduct intermediate precision. By analyzing the solution with the lowest (1 µg/mL), middle (3 µg/mL), and highest (6 µg/mL) concentration of linearity range, interday precision and intraday precision were determined. While intraday exercises were done three times on the same day, interday exercises were done three times on separate days. Following the sample's analysis, mean of area and %RSD was calculated by using peak area.

2.6.3. Accuracy

The degree of agreement between the value found and the value acknowledged as a conventional true value, or an accepted reference value is expressed as accuracy. A standard addition/standard spiking method was used for the accuracy study, and the Desidustat was spiked into the pre-quantified sample solution of the Desidustat at levels of 0, 80, 100, and 120%. Spiking was carried out into a solution containing 2 µg/mL. Three separate analyses of the entire solution were conducted. After the samples were analyzed, the data were used to calculate the area mean and the percentage of recovery.

2.6.4. Detection and Quantitation Limit

The lowest concentration of analyte in a sample that can be identified but may not always be quantified as an exact value is known as the detection limit of a particular analytical technique. The lowest concentration of analyte in a sample that can be quantitatively determined with appropriate precision and accuracy is known as the quantitation limit of a particular analytical procedure. The values of LOD and LOQ were computed in order to validate the lower range limit. The lowest limit that can be detected is known as the Limit of Detection (LOD), and it is determined by multiplying the standard deviation (σ) of the response by the slope of calibration (s), a ratio of 3.3. The lowest limit that can be both detected and quantified is known as the Limit of Quantification (LOQ). It is computed by multiplying the standard deviation (σ) of the response by ten times the slope of calibration (s).

$$\text{LOD} = 3.3 \times \frac{\sigma}{s} \qquad \text{LOQ} = 10 \times \frac{\sigma}{s}$$

2.6.5. Robustness

An analytical procedure's resilience to small, intentional changes in method parameters is measured by its robustness, which also indicates how reliable it is under typical operating conditions. Robustness was evaluated by looking at how the analysis's result changed following intentional, minor adjustments to the method's optimized parameters (wavelength, flow rate), as well as the chromatographic settings (mobile phase, mobile phase pH). By introducing each parameter change separately, the impact of the modifications on the outcome was investigated. An intermediate concentration of 3 µg/mL was employed to analyze the solution. Using the mean peak areas that were discovered through the analysis of the samples under rigorous conditions, the mean area and % RSD were computed.

2.6.6. System Suitability

Different system suitability parameters (retention time, theoretical plate, tailing factor and capacity factor) were calculated by analyzing the middle concentration (3 µg/mL) of Desidustat.

2.6.7. Analysis of Pharmaceutical Formulation

Zydus Pharmaceutical Ltd. produced 20 Oxemia-branded tablets were weighed and powdered.

After carefully weighing the powder, which equated to 10 mg of Desidustat, it was transferred to a 10 mL volumetric flask. To dissolve the contents, a fractional amount of methanol was added to the flask, and it was then sonicated for 20 minutes. Following sonication, the material was filtered through Whatman filter paper No. 41, and methanol was used as a solvent to bring the volume up to the required level, yielding a concentration of 1000 µg/mL. A 1.0 mL aliquot was taken from the above solution to dilute the content, and methanol was added to the volume to make it equal. An aliquot of 0.3 mL was taken from the above solution and diluted using a mobile phase until the solution reached the desired concentration, which was 3 µg/mL in the middle of the linearity range. The developed RP-HPLC method was used to analyze the prepared solution, and the regression equation was used to calculate the percentage amount of Desidustat.

3. Results and discussion

3.1. Selection of Analytical Wavelength

Desidustat was dissolved in methanol to produce a solution with a 10 µg/mL concentration. The solution was scanned between 400 and 200 nm in wavelength. The analytical wavelength of 230 nm was chosen because of its notable absorbance. Figure S2 in supporting information displays the UV spectra for measurand.

3.2. Optimization of Mobile Phase

The sharp symmetric peak of Desidustat at Rt of 3.02 min was produced by the mobile phase containing Methanol: Acetonitrile (80:20 v/v) pH 3.0 adjusted using 1% Orthophosphoric acid, as shown in Figure 3. And as a result, it was chosen as the mobile phase for Desidustat estimation.

3.3. Method Validation

The developed RP-HPLC method was validated as per ICH Q2 (R1) guideline and following results were obtained for defined parameters.

3.3.1. Linearity

It was found that the developed RP-HPLC method, with a correlation coefficient value of 0.9989, was linear in the range of 1-6 µg/mL. Table 1 displays the results of the regression analysis of the calibration curve. Figure 3 displays the overlay chromatogram and calibration curve for the linearity range of the calibration curve. According to the data, peak area increases linearly with concentration within the range of 1-6 µg/mL.

Table 1. Regression analysis of calibration curve of Desidustat

Parameters	**Results**
Linearity Range (µg/mL)	1 – 6
Regression Coefficient (R^2)	0.9989
Slope of regression equation	132434.33
Standard deviation of slope	583.56
Intercept of regression	4900.80
Standard deviation of intercept	1450.59

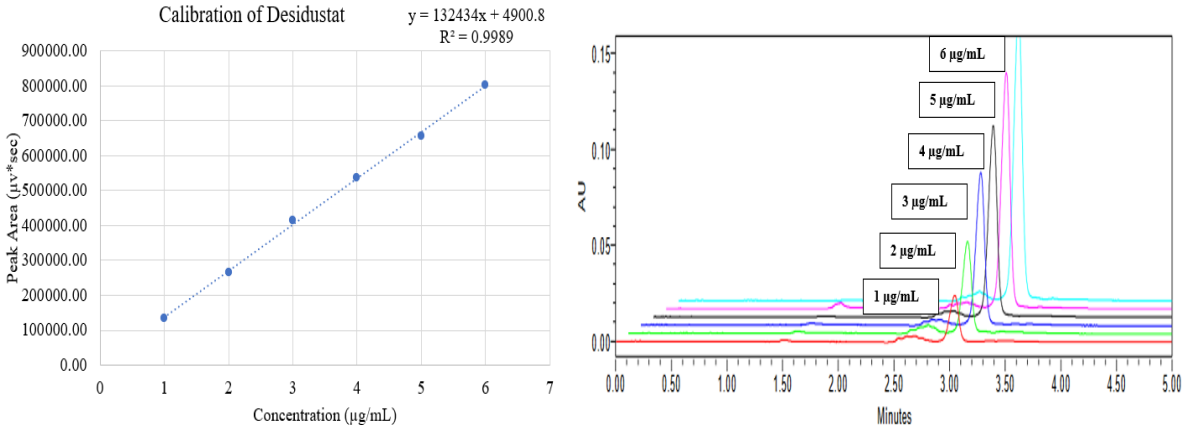

Figure 3. Calibration curve and overlay Chromatogram of Desidustat (1-6 µg/mL) using Methanol: Acetonitrile (80:20 v/v) pH 3.0 adjusted using 1% Orthophosphoric acid

3.3.2. Precision

For intraday and interday precision, the %RSD values were determined to be 0.18 – 0.95 and 0.22 – 1.25, respectively. For the repeatability study, the value of %RSD was found to be 0.25. The smaller % RSD served as confirmation of the developed method's precision. Table 2 displays the intermediate precision result. The outcome shows that the approach provides good agreement between measurements made on the same day and measurements made on different days.

Table 2. Summary of validation parameters of RP-HPLC method for Desidustat

Parameters	Result	Acceptance criteria
Linearity Range (µg/mL)	1 – 6	-
Retention Time (min)	3.02	-
Detection Limit (µg/mL)	0.04	-
Quantitation Limit (µg/mL)	0.12	-
Accuracy (% Recovery)	99.32 – 99.98	> 98% - <102%
Specificity	Specific	-
Robustness	Robust	%RSD value <2%
Precision (%RSD)		
Interday Precision (n=3)	0.22 – 1.25	%RSD value <2%
Intraday Precision (n=3)	0.18 – 0.95	%RSD value <2%
Repeatability Study (%RSD)		
Injection Repeatability (n=6)	0.25	%RSD value <2%
Assay (% Recovery)	99.04	> 98% - <102%

RSD is Relative Standard Deviation and "n" is number of determinations.

3.3.3. Accuracy

The standard addition method was used to assess the developed method's accuracy. Regression equation of calibration was used to calculate the percentage recovery of the Desidustat; the result was 99.32 – 99.98 %w/w (Table 2), indicating the accuracy of the developed method. The accuracy study's values revealed results that were closer to the true value, which is between 98% and 102%.

3.3.4. Lower Detection Limit and Lower Quantification Limit

The developed method's limit of detection (LOD) and limit of quantification (LOQ) were determined to be 0.04 and 0.12, respectively, indicating its sensitivity (Table 2). Therefore, even with limited quantity available, Desidustat can be ascertained both qualitatively and quantitatively.

3.3.5. Robustness

In order to conduct a robustness study, specific intentional modifications were made to the experimental parameters. These included changing the detection wavelength from 230 nm to 229 nm and 231 nm, the mobile phase flow rate from 1 mL/min to 0.9 mL/min and 1.1 mL/min, the mobile phase composition from 80:20 v/v to 75:25 v/v and 85:15 v/v, and the mobile phase pH from 3 to 2.8 and 3.2. Table 3 shows that the method is robust since the %RSD value was found to be less than 1% in all conditions. As a result, Desidustat can be calculated without being impacted by slight adjustments to validation parameters.

Table 3. Robustness study data of Desidustat

Parameters	Optimized Condition	Change in Condition	Mean area ± SD (n=3)	% RSD
Change in Wavelength	230 nm	229 nm	416650.0 ± 1883.8	0.45
		231 nm	414581.6 ± 1744.8	0.42
Change in Flow Rate	1 mL/min	0.9 mL/min	525241.6 ± 1524.6	0.29
		1.1 mL/min	424899.0 ± 1130.7	0.26
Change in Mobile Phase Composition	80:20 v/v	75:25 v/v	415791.0 ± 105.0	0.02
		85:15 v/v	419446.3 ± 2365.2	0.57
Change in pH of mobile phase	3	2.8	415542.6 ± 3122.3	0.75
		3.2	415241.6 ± 2630.6	0.63

3.3.6. System Suitability

The system suitability parameters result is displayed in Table 4, and values for the following parameters were directly obtained from Empower software: retention time, theoretical plate (which must be > 2000), tailing factor (which must be < 1.5), and capacity factor. Every value that was obtained fell within the range of the standard values specified in the guidelines.

Table 4. Result of System Suitability Parameters

Parameters	Result
Retention Time (min)	3.02
Theoretical Plate	2695.12
Tailing Factor (T_f)	1
Capacity Factor	1.05

3.3.7. Analysis of Pharmaceutical Formulation

The marketed tablet formulation for Desidustat was analyzed using the suggested method, and the results indicated a drug recovery percentage of 99.04 %w/w, indicating the method's applicability. Figure 4 displays the overlay chromatogram for the Desidustat standard and formulation along with its selectivity and specificity.

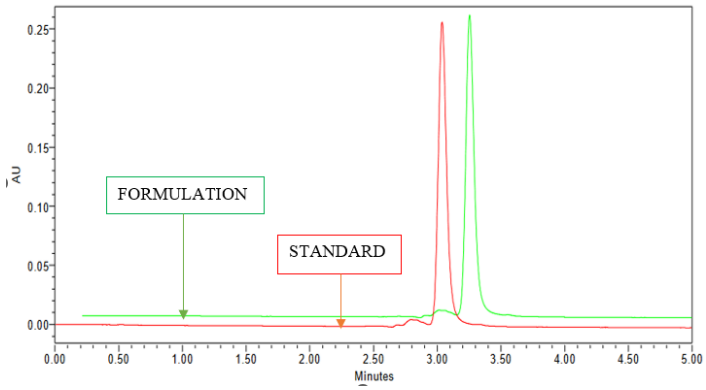

Figure 4. Overlay chromatogram of Standard Desidustat and Marketed Formulation of Desidustat (10 µg/mL of Desidustat in both) using Methanol: Acetonitrile (80:20 v/v) pH 3.0 adjusted using 1% Orthophosphoric acid.

3.4. Uncertainty Assessment

The uncertainty assessment for method was done as per the EURACHEM/CITAC guide and the corresponding literatures [17-23]. We have reported the combined uncertainty ($u_{combined}$) and expanded uncertainty ($U_{Expanded}$), calculated from the uncertainty in standard preparation ($u_{standard}$), uncertainty associated with the slope of calibration curve ($u_{calibration}$), uncertainty of recovery ($u_{recovery}$) and uncertainty of repeatability ($u_{repeatability}$) using following equation 1.

$$u_{combined} = \sqrt{(u_{standard})^2 + (u_{calibration})^2 + (u_{recovery})^2 + (u_{repeatability})^2} \quad (1)$$

$U_{standard}$ of analyte was calculated from the %purity provided by the supplier using equation 2.

$$u_{standard} = 100 - \%Purity/\sqrt{3} \quad (2)$$

$U_{calibration}$ was calculated for analyte from the standard error of slope and slope value for the calibration curve using equation 3.

$$u_{calibration} = (\text{Standard Error of Slope} * 100)/\text{ Slope} \quad (3)$$

The mean relative standard deviation (RSD) associated with the recovery studies was considered as $U_{recovery}$ while that of repeatability studies was considered as $U_{repeatability}$ for the analyte. Expanded uncertainty at a 95% confidence interval is calculated by multiplying combined uncertainty with the coverage factor (k = 2) The uncertainty profile for the present method is given in table 5.

Table 5. Uncertainty Assessment of Reverse phase-HPLC method

Sources	Uncertainty
$u_{standard}$	0.15
$u_{calibration}$	2.69
$u_{recovery}$	0.14
$u_{repeatability}$	0.18
$u_{combined}$	1.78
$U_{expanded}$	3.56

$U_{Expanded}$: k=2 95 % confidence level; U % values reported

3.5. Comparison with Reported Methods

Presented method shows sensitive linearity range of 1-6 µg/mL with less retention time of 3.02 as compared to other reported papers.

Table 6. Comparison of HPTLC and RP-HPLC for determination of Desidustat

Sr. No.	Desidustat	
	% Amount of drug found	
	HPTLC	RP-HPLC
1	99.08	98.98
2	99.33	99.10
3	99.19	99.04
Mean	99.20	99.04
SD	0.08	0.24
F_{cal}	9.00	
F_{tab}	9.28	

Table 7. Comparison among published analytical methods and current method [8,9,10]

Sr. No.	Parameters	HPTLC method [8]	RP HPLC method[9]	RP HPLC method [10]	This study
1	Stationary phase	Precoated silica gel 60 F254	Column C_{18} (25cm × 0.46 cm) Hypersil BDS	Thermofisher C_{18} (150mm*4.6mm) 5µm	C_{18} Hypersil (250 x 4.6 mm, 5µm)
2	Mobile phase	Toluene: Methanol: Glacial acetic acid (7.5:2.5:0.3 v/v/v)	Methanol: Acetonitrile (70:30 v/v) with pH 4	ACN: 0.1% Formic Acid (80:20 v/v)	Methanol: Acetonitrile (80:20 v/v) pH 3 with OPA
3	Linearity range	100-600 ng/band	3-7 µg/mL	-	1-6 µg/mL
4	Retention time	-	9.52	-	3.02
5	R_f value	0.65	-	-	-
6	Regression Coefficient (R^2)	0.9979	0.9992	0.999	0.9989
7	LOD		0.165 µg/mL	-	0.04 µg/mL
8	LOQ		0.539 µg/mL	-	0.12 µg/mL
	System suitability parameters				
1	Theoretical Plate	-	Not reported	-	2695.12
2	Tailing Factor (T_f)	-	Not reported	-	1
3	Capacity Factor	-	Not reported	-	1.05

The comparison of published analytical techniques and the RP HPLC method for Desidustat estimation is displayed in the above table. The data indicates that the linearity range of the RP HPLC method is 1-6 µg/mL, while the stability indicates a range of 3-7 µg/mL. More retention time was taken into account for the estimation of degradant peaks in the stability RP HPLC methods that were reported. Furthermore, the RP HPLC method exhibits greater sensitivity in terms of lower quantification limits of 0.12 µg/mL and lower detection limits of 0.04 µg/mL. Three parameters are included in the system

suitability data representation: the theoretical plate value 2695.12 (>2000), the tailing factor 1, and the capacity factor 1.05.

4. Conclusion

Using C18 Hypersil (250 x 4.6 mm, 5µm) as the stationary phase and methanol: acetonitrile (80:20 v/v) as the mobile phase, a reverse phase high performance liquid chromatographic method to quantify Desidustat has been developed. For Desidustat, the developed method was found to be linear in the range of 1-6 µg/mL with a retention time of 3.02 minutes. Desidustat's % recovery was determined to be 99.32–99.98%. Sensitivity of the method to detect Desidustat in trace amounts is indicated by low concentration values of the quantitation and detection limits. For routine Desidustat analysis, the method was found to be precise, accurate, specific, repeatable, and reproducible. Therefore, without excipient interference, the method described here can be used for quantification of Desidustat in both bulk and tablet dosage form.

Acknowledgements

All the authors are thankful to the Indukaka Ipcowala College of Pharmacy and Sophisticated Instrumentation Centre for Applied Research and Testing (SICART) for providing the facility and necessary help to conduct an experimental work.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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