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Original Article

Analytical Method Development and Validation by RP-HPLC and UV Spectrophotometric for Estimation of Daprodustat in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

Objectives: This research aimed to systematically boost the development and validation of newly devised, precise, reproducible and accurate UV and Reverse Phase High Performance Liquid Chromatography (RP-HPLC) methods for Daprodustat. **Materials and Methods:** UV-visible spectrophotometer (Jasco V-730) was used for the analysis and the absorbance was measured across the UV and visible spectra. Measurements were taken at 265 nm, a UV wavelength. Samples were prepared in methanol and selected for compatibility with both UV-vis spectroscopy and the analytes being studied. HPLC-Chromatographic separation was accomplished on a Waters C18 column [150×4.6 mm, 5 µm]. The mobile phase was 0.1% perchloric acid in water: acetonitrile (50:50, V/V), the flow rate was set at 1.0 mL/min and the detector wavelength was 265 nm utilizing the UV detector coupled with an integrator program and data recorder. **Results:** Daprodustat had a retention time of 15.8 min. With a correlation coefficient of 0.999, the linearity concentration varied from 5-25 µg/mL (HPLC) and 2-10 µg/mL (UV). The accuracy ranged between 98.25%-101.89% (HPLC) and 100.91%-101.40% (UV). In this study, observed that the UV method yielded RSDs with precision less than 2% for both intraday (1.43%) and interday (1.20%) analyses. Similarly, the HPLC method demonstrated system precision (1.56%) and method precision (1.52%) below 2%. **Conclusion:** Successfully developed and validated the HPLC and UV spectrophotometric techniques in compliance with the specificity, precision, linearity, accuracy, LOD, LOQ and robustness guidelines outlined by the International Council for Harmonization (ICH). Subsequently, the method was applied for routine analysis purposes.

Keywords: Daprodustat, RP-HPLC, UV-Spectroscopy, Validation, Analytical Method.

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INTRODUCTION

Progressive chronic renal failure is linked to significant health issues such as anemia, cardiovascular disease risk, hyperlipidaemia and metabolic bone disease.[1] In Chronic Kidney Disease (CKD), the primary cause of anemia insufficient production of Erythropoietin (EPO) on diseased kidneys due to hypoxia or anemia.[2] Infection, inflammation and a lack of vitamins and iron are other factors that can lead to hemophilia.[3] Hypoxia-Inducible Factor 1-alpha and Hypoxia-Inducible Factor 2-alpha, as transcription factors, work together to regulate gene expression in distinct cells during hypoxia. Kidneys and livers boost EPO production, triggering erythropoiesis and enhancing iron transport.[2-4] A family of enzymes known as HIF-Prolyl Hydroxylases (PHDs) is responsible for controlling HIFα levels and maintaining the equilibrium between HIF activity and oxygen availability.[5] When PHDs are inhibited, mildly hypoxic environment is stimulated by preventing HIFα breakdown, leading to an erythropoietic reaction.[5] PHDs are attractive targets for treating anemia due to their crucial role as enzymatic gatekeepers in the physiological reaction to hypoxia.[6]

Daprodustat, a small molecule HIF prolylhydroxylase inhibitor, received approval in the US and Japan in 2023 and 2020, respectively, for treating anaemia in patients with chronic kidney disease.[7-10] It selectively inhibits PHD 1-3 with greater than 1000-fold specificity.[11] The liver primarily metabolizes PHD 1-3, with their metabolites excreted in urine and feces.[12] Tablets containing Daprodustat are sold under the trade name Jesduvroq (GlaxoSmithKline).

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Monica Mazzarino, Ilaria Perretti, *et al.*, (2021)[13] focus on estimating Prolyl-Hydroxylase Inhibitors of HIF in urine using ultra-performance liquid chromatography-mass spectrometry procedures. UPLC, along with high- or low-resolution MS, was used for the instrumental analysis. In this article, they employed A Supelco Ascentis® C18 column (150×2.1 mm, 2.7 µm) was utilize (Sigma-Aldrich, Milano, Italy). Eluent A, which is ultra-purified water and eluent B, which is acetonitrile with 0.1% formic acid, were chosen as the mobile phases. Using a gradient program, elution started at 5% eluent B, increased to 65% in 7 min and reached 100% in 1 min for 4 min. After the gradient was applied, re-equilibration was performed at 5% for 2 min. The column temperature was 30°C and the flow rate was 250 µL/min. The injection volume was 10 µL. According to the literature review, no technique for estimating Daprodustat in bulk or pharmaceutical dose forms has been described thus far, except for a few bioanalytical approaches.

Analytical procedures evolve to meet changing requirements, ensuring simplicity, reliability, cost-effectiveness, reproducibility and accuracy. The work intended to provide a reliable, precise, sensitive and reproducible gradient RP-HPLC technique for determining Daprodustat in dose forms such as tablet and in bulk. As the demand for effective and innovative medications continues to rise, the need for robust analytical methods to ensure the quality, safety and efficacy of these compounds becomes paramount. This manuscript comprehensively explores the development and validation of an analytical method for Daprodustat, shedding light on the meticulous processes undertaken to determine reliable protocols for its quantitative analysis. The significance of this research lies in its contribution to the pharmaceutical sciences, as accurate and precise analytical methods are essential not only for quality control during drug manufacturing, but also for pharmacokinetic studies, bioequivalence assessments and clinical trials. Furthermore, the validation aspects covered in this manuscript provide a broad grasp of the reliability and robustness of the analytical methods employed. The validation parameters, including specificity, linearity, precision, accuracy, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ), were comprehensively evaluated, guaranteeing adherence to the rigorous regulatory standards set forth by health authorities and ICH guidelines.[14] Here is the drug profile: The compound, with the IUPAC name 2-[(1,3-dicyclohexyl-2,4,6-trioxo-1,3-diazinane-5-carbonyl)amino] acetic acid, has a molecular formula of C19H27N3O6 and a molar mass of 393.440 g·mol-1. It belongs to the category of hypoxia-inducible factor prolyl hydroxylase inhibitors. This white to off-white powder exhibits insolubility in water but shows solubility in methanol and acetonitrile.[15,16]

MATERIALS AND METHODS

Instrumentation

An Agilent 1100 series high-performance liquid chromatography that runs on lab solution software and features a quaternary gradient pump, an autosampler and a UV detector with adaptable wavelength capability linked to a data recorder. A Sartorius analytical balance was employed for the precise weighing and preparation of samples. The dimension of the Waters C18 column used for chromatographic separation were 150 cm×4.6 mm×5 µm. In this study, a UV-visible spectrophotometer, namely the Jasco V-730 model, was utilized. Using a UV-visible double-beam spectrophotometer, the instrument was outfitted with matched quartz cells measuring 1 cm and the results were analysed with UV-PROBE software.

Reagents and chemicals

High-quality, HPLC-grade reagents and solvents were used in this study. Acetonitrile, methanol and perchloric acid were provided by Finar Ltd., Mumbai, India, for use in the study. The Milli-Q system is a water purification system based on Reverse Osmosis (RO). The Research laboratory Finechem, located in Mumbai, supplied 0.45 µm nylon filters, which were utilized for filtration. The accuracy and reliability of HPLC analysis enhances using quality reagents, solvents and filters.

Chromatographic conditions

The gradient elution method was used to conduct the experiments. The mobile Phase composition gradually increased during the elution process, but regularly changed during step elution. The mobile phase contained 0.1% aqueous perchloric acid and acetonitrile at a 50:50 ratio. To eliminate any particles, the mixture was passed through a membrane filter. The mobile phase was degassed eliminate any dissolved gases that might have interfered with the analysis before the samples were run. Throughout the HPLC analysis, the flow rate of the mobile phase was maintained at 1 mL/min. The column temperature was held constant at 30°C, corresponding to the ambient temperature, during chromatographic separation. A UV detector with a wavelength of 265 nm (Figure 1) was used to detect the effluents from the column after a 10 µL volume of sample was injected per HPLC run. Acknowledging the unique UV absorption characteristics of Daprodustat, this specific wavelength was chosen for detection. The HPLC system was able to efficiently separate and quantify Daprodustat in the sample by using these experimental conditions, resulting in reliable and accurate results for analysis (Table 1).

Selection of the mobile phase

To achieve a well-resolved, distinct and symmetrical peak for the drug, various solvents were used. The standard solution preparation and the chromatographic conditions for analysis were

consistent with those previously outlined, with adjustments made to the mobile phase composition as appropriate. The number of theoretical plates, proper separation, peak purity index and other considerations were used to determine the mobile phase. The mobile phases detailed in the Table 2 underwent testing.

Buffer preparation

1 mL of perchloric acid was diluted in 1000 mL of HPLC-grade water. The resulting mixture was filtered through a nylon membrane filter with a diameter of 0.45 µm. Subsequently, the filtered solution was degassed to eliminate any dissolved gases.

Preparation of solutions

Preparation of Blank

The diluent was used as a blank.

Preparation of standard solution stock

10 mL of the working standard were accurately weighed into a 10 mL volumetric flask. Add 5 mL of diluent, thoroughly shake the flask and then use the diluent to reach the mark, resulting in a solution of 1000 µg/mL. From this solution, 1 mL was pippetted into a 10 mL volumetric flask and diluted with the diluent until the solution reached a concentration of 100 µg/mL.

Preparation of sample solution stock

Ten tablets were weighted accurately and powdered. After the powder was meticulously measured to match the weight equivalent to 10 mg of Daprodustat tablet powder, the powder was transferred to a 10 mL volumetric flask. Add 5 mL of solvent, was added, the mixture was shaken thoroughly and then, sufficient solvent was added to reach the 1000 µg/mL mark. From this solution, 1 mL was pippetted out and transferred to a 10 mL volumetric flask, after which the solution was diluted to 100 µg/mL.

Assay Method

Preparation of Standard Solution

Approximately 10 mg standard drug weighed and transferred to a 10 mL volumetric flask. Add 5 mL of diluent and sonicate to facilitate dissolution. The solvent was subsequently used to adjust the volume to the mark, yielding a solution with a concentration of 1000 µg/mL. Subsequently, 1 mL of the aforementioned solution pippetted out and transferred to a 10 mL volumetric flask. The mixture was diluted with the solvent until the concentration reaches the 100 µg/mL.

Preparation of sample solution

Ten tablets were weighed accurately and powdered. After the powder was measured to achieve an equivalent of 10 mg of Daprodustat powder, the powder was transferred to a 10 mL flask. Add 5 mL of solvent, was added the mixture was shaken well and then sufficient solvent was added to reach the 1000 µg/mL mark. From this solution, 1 mL was pippetted into a 10 mL volumetric flask and diluted to a concentration of 100 µg/mL. The dosage form was determined to contain 100.07% of Daprodustat (Table 3).

Validation Parameters (By HPLC)

Specificity and selectivity

A specific analytical technique must be used to accurately detect chemicals in a sample while avoiding disturbance from other compounds, distinguishing the target substance from potential interferences and revealing a unique approach through chromatographic comparison. The Figures display the chromatograms of the standard and blank injections (Table 4). Hence, the method was found to be Specific, as shown in Figure 2.

Linearity

A standard stock solution was diluted to evaluate the linearity of the daprodustat solution at concentration ranging from 5-25 µg/

Table 1: Chromatographic conditions.

Chromatographic conditions	
Specification	**Comment**
Column	Waters C18 (150×4.6 mm, 5 µM) dimension.
Detection Wavelength	265 nm
Flow rate	1 mL/min
Column Temperature	30°C
Sample Temperature	10°C
Injection volume	10 µL
Run time	20 min
Mobile Phase	Mobile Phase composition: Mobile phase (A): 0.1% PCA in water Mobile phase (B): ACN [50:50].
Diluent	Water: Methanol (05:95, v/v).
Seal Wash	Acetonitrile: Water [20:80, v/v].
Needle Wash	H2O: Methanol (10:90, v/v).
Elusion mode	Gradient Program- Time (min) %M.P.-A %M.P.-B 0.01 50 50 05 95 05 10 20 80 14 20 80 16 50 50 20 50 50

mL. The peak area responses determined the drug concentration linearity through a plotted graph, which demonstrated that a linear association existed between the concentration of the drug and the area response within a range. A Table 5 and Figures 3, 4 show the findings that were achieved.

Precision

The system and method precision were evaluated by injecting a 15 µg/mL sample six times for system precision and by injecting a standard solution six times for method precision. The RSD for standard preparation was low at 1.52%, indicating the accuracy of the HPLC system. Sample preparation yielded a similar RSD of 1.56%, confirming its accuracy. The method proved to be accurate after both preparations were applied. The method precision was within the acceptable 2.0% limit (Table 6).

Accuracy

Recovery investigations were performed in triplicate using 3 different injection concentrations (80%, 100% and 120% Daprodustat). Results are in line with expected values that fall within the permissible range, as the Table 7 with spiking concentrations and recovery percentages illustrates. The effective recovery at various spiking levels validates the accuracy of the method, rendering it reliable for quantitative analysis.

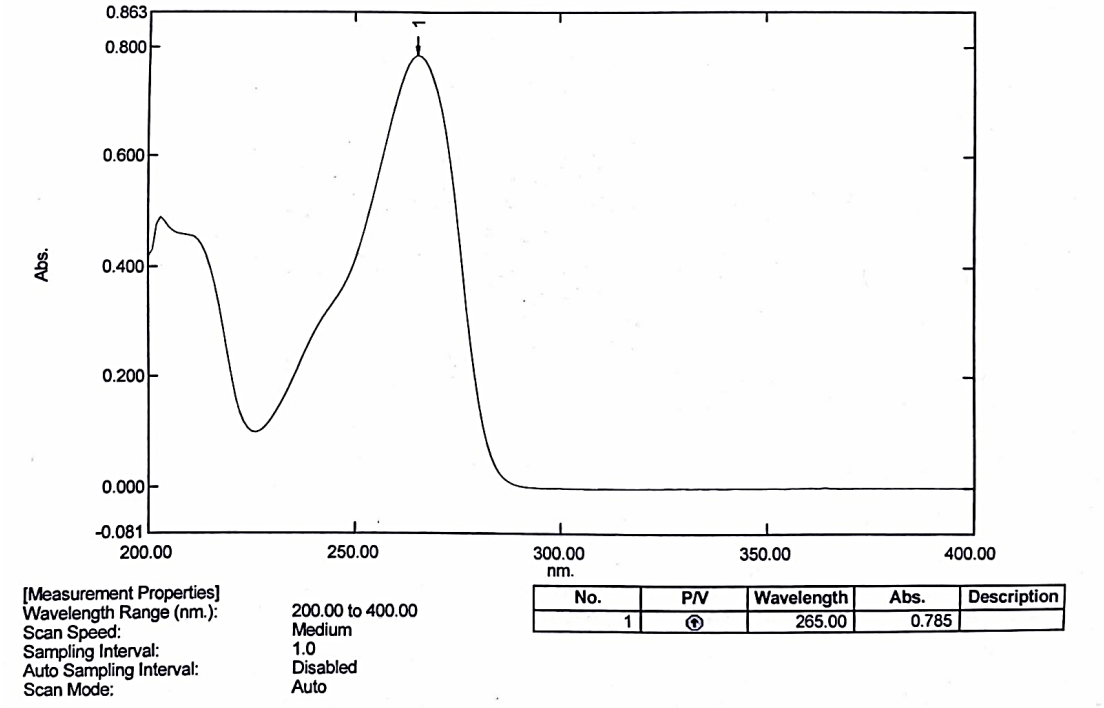

Figure 1: UV spectrum of daprodustat.

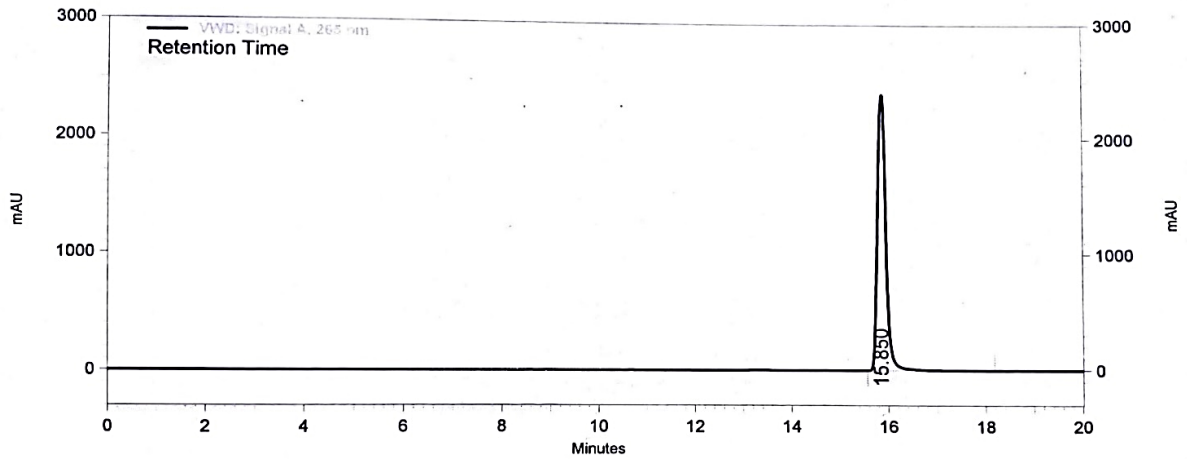

Figure 2: Chromatogram of standard solution for specificity.

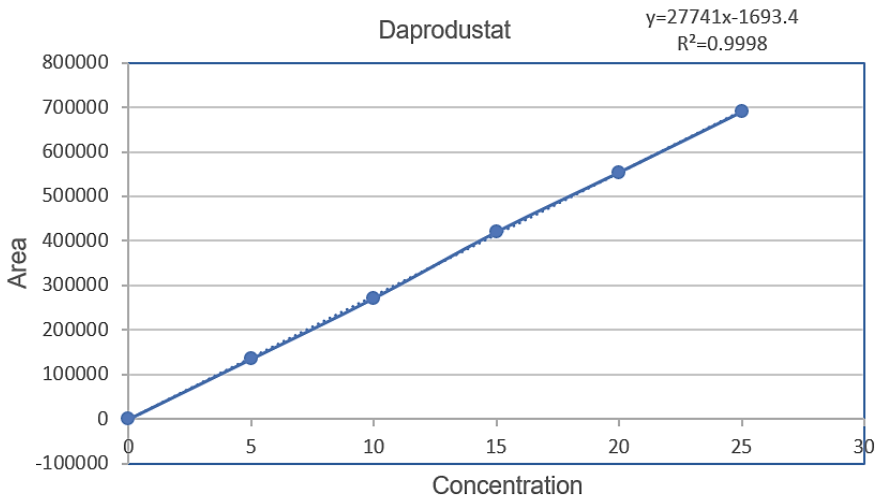

Figure 3: Calibration curve generated by HPLC- Daprodustat.

Table 2: List of mobile phase compositions screened.

Trial No.	Mobile Phase composition	Wavelength	pH	Comment	Observation
1	Buffer: ACN (50:50 v/v)	265 nm	3	Tailing and fronting.	Not satisfactory
2	ACN: H_2O (50:50 v/v)		-	Noisy Peak with improper baseline.	Not satisfactory
3	(ACN: MEOH): H_2O (25:25:50 v/v)		-	Noisy Peak	Not satisfactory
4	MEOH: H_2O (50:50 v/v)		-	Peak not observed.	Not satisfactory
5	OPA: ACN (50:50 v/v)		-	Tailing	Not satisfactory
6	OPA: MEOH (50:50 v/v)		-	Peak not observed.	Not satisfactory
7	Formic acid: Acetonitrile (50:50 v/v)		-	Broad peak	Not satisfactory
8	Perchloric acid: ACN (50:50 v/v)		-	Sharp peak with good resolution.	Satisfactory

Table 3: Results of the Daprodustat Assay via RP-HPLC.

Sample ID	Concentration [µg/mL]	Area [Standard]	Area [Sample]
1	10	271520	271450
2	10	271525	271455
3	10	271540	271460
4	10	271530	271465
5	10	271532	271470
6	10	271545	271478
	Average	271532	271463
	Std. Purity	100	
	Assay (%)	100.07	

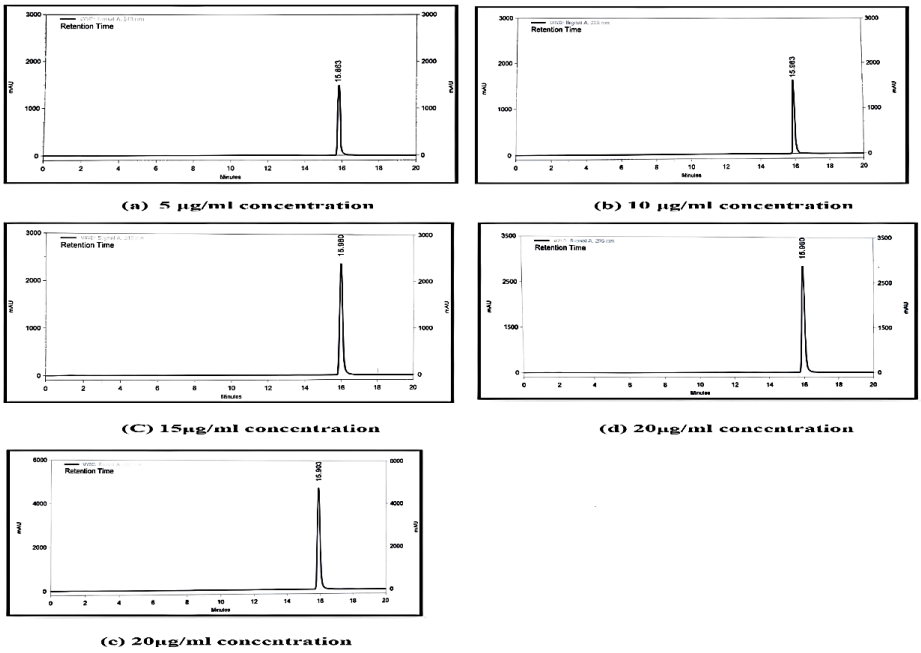

Figure 4: Chromatograms of daprodustat for linearity.

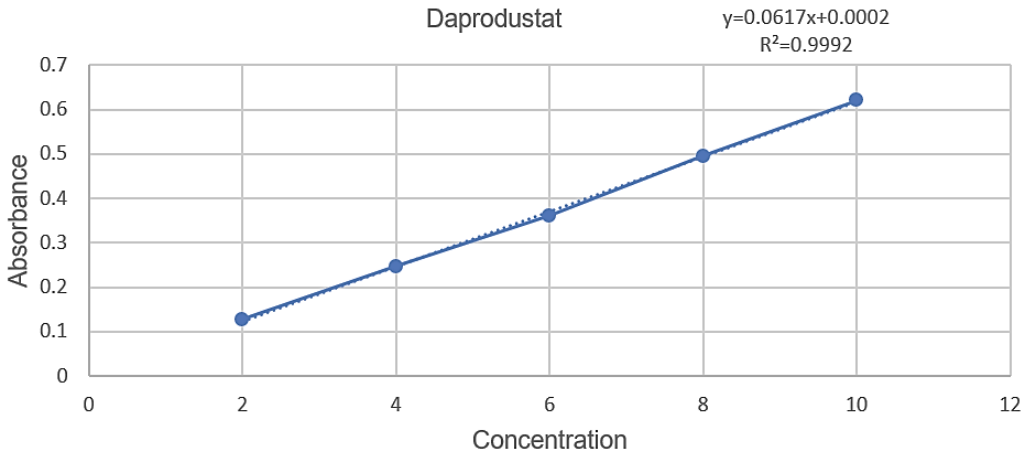

Figure 5: Calibration curve by UV-Daprodustat.

System suitability

System suitability tests confirmed the reliable of the HPLC system by analyzing the reference solution at a concentration of 1000 µg/mL in six identical injections. Parameters such as the tailing factor, plate count and column efficiency were noted for each injection and compared against predefined limits. The recorded data showed that the system operated effectively within acceptable levels, as displayed in the results Table 6.

Limit of Detection (LOD)

The lowest detectable concentration is known as the Limit of Detection (LOD).

Was calculated as LOD=3.3*(N/S). The determined LOD was 0.3484 µg/mL, where S is the slope of the calibration curve and N is the standard deviation of the peak area.

Table 4: Specificity parameters of the daprodustat.

Peak Name	Daprodustat	Acceptance criteria
Retention Time (RT)	15.85 min	-----
Area	421839.5	-----
Area%	100.00	-----
Theoretical plates (N)	45850	Not Less than 2000
Tailing Factor (T)	1.29	Not more than 2.0

Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) is the lowest detectable concentration that cannot always be measured. It is computed utilizing the formula:

LOQ=10*(N/S)

where S is the slope of the calibration curve and N is the standard deviation of the peak area, resulting in a determined LOQ of 1.0557 µg/mL.

Robustness

The flow rate and wavelength were varied to test the stability of the HPLC method. The of the method resistance to small changes was demonstrated by the results, which showed no apparent changes in the chromatogram or other characteristics. These findings prove the method's ability to maintain accuracy under varied conditions.

The table summarizes the results of the robustness tests, which included flow rate ±0.2 mL/min, temperature ±5°C and Wavelength ±2 nm. Acceptance criteria: Require % RSD less than 2.0%.

Conclusion: Parameters met the criteria that % RSD was consistently less than 2.0% under all the variable conditions tested Table 8.

UV-visible Spectrophotometric Methods

Specificity

The reference standard ensured specificity and no interference occurred.

- Blank solution to show no interference and,
- Sample solution to show no interference with excipients.

Table 5: Linearity of Daprodustat (HPLC).

Injection	Concentration (µg/mL)	Peak Area
Injection 1	0	0
Injection 2	5	135022
Injection 3	10	271339
Injection 4	15	420298
Injection 5	20	553969
Injection 6	25	689765
Correlation Coefficient		0.9998
Y-Intercept (c)		-1693.4
Slope (m)		27741
Regression Equation		Y=27741x-1693.4

Table 6: Precision and System suitability Data for Daprodustat obtained via the RP-HPLC method.

Standard number	RT (min)	Concentration (µg/mL)	Peak Area		
			[System]	[Method]	System Suitability
1	15.8	15	413701	415791	411713
2	15.8	15	425488	413436	427431
3	15.8	15	424390	425350	429281
4	15.8	15	424417	427210	421808
5	15.8	15	412336	429488	418991
6	15.8	15	427891	423651	421813
Mean			421370.5	422487.7	421839.5
%RSD			1.56	1.52	1.48

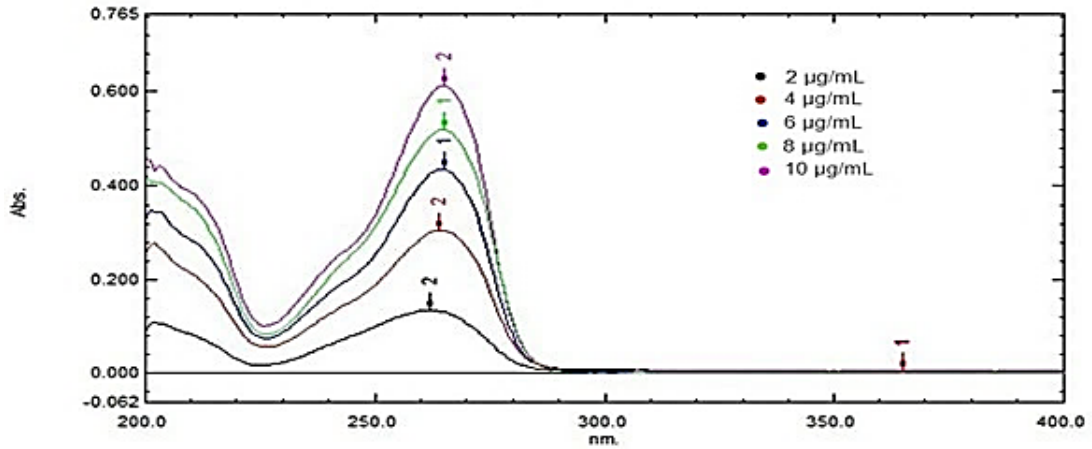

Figure 6: Overlay spectra by UV-Daprodustat.

Table 7: Accuracy Data RP-HPLC Method.

Level	Sample Concentration	Standard Concentration	Total Concentration	Peak Area	Mean	% Recovery
80%	10	8	18	489036	489038	98.25
	10	8	18	489039		
	10	8	18	489041		
100%	10	10	20	553950	553957	100.15
	10	10	20	553960		
	10	10	20	553962		
120%	10	12	22	620115	620114	101.89
	10	12	22	620110		
	10	12	22	620118		

Table 8: Data of Robustness for Daprodustat by RP-HPLC Method.

Sr. No.			I	II	III	Mean	Standard Deviation	%RSD
Flow Rate±0.5 mL/min	0.95 mL/min	Area	424243	423479	424226	423983	436.27	0.10
		Rt	16.07	16.07	16.07	16	0.01	0.04
	1.05 mL/min	Area	425648	422824	425223	424565	1522.65	0.36
		Rt	16.15	16.15	16.15	16	0.00	0.00
Temperature±5°C	25°C	Area	427522	426411	425828	426587	860.61	0.20
		Rt	15.99	15.98	15.98	16	0.01	0.04
	35°C	Area	427148	426242	425223	426204	963.05	0.23
		Rt	15.99	15.98	15.98	16	0.01	0.04
Wavelength±2 nm	263 nm	Area	422548	421446	420148	421381	1201.33	0.29
		Rt	15.98	15.98	16	16	0.01	0.07
	267 nm	Area	422011	421005	422311	421776	684.07	0.16
		Rt	16.08	16.07	16.12	16	0.03	0.16

Linearity

Standard solutions of daprodustat (2-10 µg/mL) were prepared for linearity assessment, generating a calibration curve (Figure 5) through regression analysis. Representation of Overlay spectra of linearity by UV-Daprodustat is as shown in Figure 6. The results of the Daprodustat linearity by HPLC method are presented in Table 9.

Table 9: Linearity of Daprodustat (UV).

Concentration (µg/mL)	Absorbance
2	0.128
4	0.247
6	0.361
8	0.495
10	0.621
Correlation Coefficient	0.9992
Y-Intercept (c)	-0.0002
Slope (m)	0.0617
Regression Equation	Y=0.0617x+0.0002
% RSD	0.5269

Accuracy

Accuracy, also known as a recovery study, is conducted to assess accuracy parameters at three different concentration levels. Known amounts of daprodustat were added to pre-analyzed samples. These studies were carried out three times to calculate the recovery percentage as indicated in Table 10.

Precision

Precision is used to calculate or monitor the repeatability of a test solution (Table 11).

Interday

Six replicates of a 10 µg/mL standard solution were prepared. The absorbance was measured twice daily, used solvent as a blank in the reference cell. The %RSD of the absorbance was calculated.

Intraday

Six replicates of a 10 µg/mL standard solution were prepared and the absorbance was measured on different days using solvent in the reference cell as a blank. The %RSD of the absorbance was calculated.

Robustness

10 g/mL standard solution was prepared and the absorbances were calculated with the solvent as a blank. The wavelength was varied to check the system suitability. The results were shown in Table 12.

LOD and LOQ

According to ICH guidelines, the limit of detection and limit of quantitation were calculated with one of the following formulas based on the response and slope of the regression equation after six sets of linear concentrations were analyzed.

LOD=3.3xc/s

LOQ=10xc/s

Where, 'c'=standard deviation and 's'=slope.

Table 10: Accuracy data for daprodustat by UV Method.

Level	Sample Concentration	Standard Concentration	Total Concentration	Absorbance	Mean	% Recovery
80%	4	3.2	7.2	0.447	0.448	100.91
	4	3.2	7.2	0.451		
	4	3.2	7.2	0.448		
100%	4	4	8	0.498	0.500	101.32
	4	4	8	0.501		
	4	4	8	0.502		
120%	4	4.8	8.8	0.550	0.551	101.44
	4	4.8	8.8	0.551		
	4	4.8	8.8	0.552		

Table 11: Precision data for Daprodustat [UV].

Injection	Concentration(ug/mL)	Interday [1]	Interday [2]	Intraday
		Absorbance		
INJ 1	10	0.629	0.634	0.631
INJ 2	10	0.638	0.626	0.621
INJ 3	10	0.634	0.637	0.636
INJ 4	10	0.621	0.627	0.635
INJ 5	10	0.633	0.642	0.648
INJ 6	10	0.619	0.641	0.641
Mean		0.629	0.635	0.635
Standard deviation		0.007563	0.006833	0.009136
%RSD		1.2023	1.0770	1.4379

Table 12: Robustness data of Daprodustat by UV Method.

Injection	Concentration	Absorbance/wavelength		
		263	265	267
1	10	0.608	0.615	0.601
2	10	0.610	0.618	0.606
3	10	0.612	0.619	0.605
SD		0.005	0.005	0.006
%RSD		0.814	0.804	0.985

The limit of detection was found to be 0.3599 µg/mL, while the limit of quantitation was determined to be 1.0907 µg/mL.

CONCLUSION

During the optimization of the RP-HPLC method for Daprodustat analysis, various mobile phase compositions were tested and the combination of acetonitrile and perchloric acid yielded the best results. A wavelength of 265 nm was chosen for detection due to the significant UV absorption signal of Daprodustat. The resulting method achieved a retention time of 15.8 min, providing well-separated and identifiable peaks. Overall, the developed HPLC method meets the requirements for quick analysis time and resolution, with accurate calibration in the 5-25 µg/mL range. The limit of detection and limit of quantitation were determined to be 0.3484 µg/mL and 1.0557 µg/mL respectively, demonstrating the sensitivity of the method. The system precision, method accuracy and recovery percentages for Daprodustat fell within acceptable ranges. The method showed resilience to parameter changes, maintaining consistent results with improved efficiency and accuracy in Daprodustat measurements.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UV: Ultraviolet; **RP HPLC:** Reverse Phase High Performance Liquid Chromatography; **SD:** Standard deviation; **RSD:** Relative standard deviation; **LOQ:** Limit of quantitation; **LOD:** Limit of detection; **INJ:** Injection; **V/V:** Volume by volume; **NTP:** Normal Temperature and Pressure; **Rt:** Retention time; **Min:** Minutes; **µg/mL:** Microgram per millilitre; **nm:** Nano meter.

SUMMARY

Successfully developed and validated the HPLC and UV spectrophotometric techniques for estimation of daprodustat in bulk and pharmaceutical dosage form in compliance with the specificity, precision, linearity, accuracy, LOD, LOQ and robustness guidelines outlined by the International Council for Harmonization (ICH). subsequently, the method was applied for routine analysis purposes.

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