Ind. J. Pharm. Edu. Res., 2024; 58(2s):s532-s542.
https://www.ijper.org

Original Article

A Validated Simple, Rapid and Accurate RP-HPLC Approach for Measuring Vardenafil Hydrochloride Trihydrate in Bulk Drugs and Medicinal Formulations

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ABSTRACT

Background: Shortcomings in current analytical methods characterized by time-consuming procedures, limited sensitivity, and high costs associated with reagents and instruments, influence the research background. **Objectives:** The goal of this research was to find a simple, fast, and accurate way to discern and quantify substances in bulk drugs and finished products at the utmost minimal levels of detection and quantification. **Materials and Methods:** The methodology involved using a C_{18} column then a solvent mixture comprising 0.1% orthophosphoric acid and acetonitrile in an 80:20 proportion delivered at 1 mL/min^{-1} and observed at 247 nm with a UV detector. **Results:** Following rigorous verification in line with ICH guidelines, the technique revealed several notable features. It revealed rapidity with a retention time of 2.53 min, accurate with average recoveries ranging from 99.20% to 100.43%, precision as demonstrated by a relative standard deviation below 2%, specificity, and a highly linear correlation coefficient of 0.999 between 0.0025 to 6 µg mL^{-1}. Additionally, the method displayed remarkable sensitivity, detecting concentrations as low as 56.91 ng mL^{-1} and a quantification limit of 172.44 ng mL^{-1}. **Conclusion:** This tested RP-HPLC method has a unique mix of speed, accuracy, and high sensitivity that makes it possible to accurately measure Vardenafil hydrochloride trihydrate, even at nanogram levels. This surpasses established techniques and offers a cost-effective solution for pharmaceutical quality control and analysis.

Keywords: RP-HPLC, Vardenafil hydrochloride Trihydrate, LOD, Nanogram.

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Received: 09-03-2023;
Revised: 04-11-2023;
Accepted: 07-03-2024.

INTRODUCTION

Vardenafil Hydrochloride Trihydrate (VHT) is a chemical compound called Piperazine, 1-[[3-(1,4-dihydro-5-methyl-4-oxo-7-propylimidazo [5, 1-f] [1, 2, 4] triazin-2-yl)-4-ethoxyphenyl] sulfonyl] -4-ethyl-, monohydrochloride trihydrate. It inhibits phosphodiesterase type 5, which only breaks down cGMP with great potency and selectivity. Basically, it appears as a white or light brown or yellowish powder having a molecular mass of 579.1 g/mole and a molecular formula of $C_{23}H_{33}ClN_6O_4S$, $3H_2O$, as depicted in Figure 1.

The original form of VHT, Vardenafil (VDF), was created with the goal of providing a safe and efficacious oral treatment for erection problems in men.[1] Extensive research has revealed that VDF displays considerably enhanced performance, being 5-10 times more effective compared to sildenafil, which is a common PDE-5 inhibitor. It acts by facilitating smooth muscle relaxation, which is crucial in achieving an erection.[2] It selectively blocks the phosphodiesterase-5 enzyme, which metabolizes cyclic guanosine monophosphate, thereby impeding its conversion into inactive GMP. This mechanism ultimately elevates cGMP levels in the bloodstream, resulting in smooth muscle relaxation and dilation. Apart from its function in addressing erectile dysfunction, VDF exhibits promise in the treatment of several cardiovascular ailments, including Pulmonary Arterial Hypertension (PAH).[3] Notably, VDF has emerges as a viable and inexpensive primary treatment option, particularly in developing nations. Its affordability, coupled with its capability to enhance blood flow, exercise capacity, and treatment outcomes, renders VDF an advantageous therapeutic approach for individuals with PAH who have never had it before.[4]

To date, array of techniques have been employed to quantify VDF, either alone or in mixture with other PDE-5 inhibitors in

DOI: 10.5530/ijper.58.2s.55

pharmaceutical formulations. These techniques include UV/VIS,[5-6] LC-ESI-MS,[7-9] LC-MS/MS,[7,8,10-14] GC-MS,[15] UPLC-DAD,[16] UPLC-TOF,[17] HPLC-UV (human plasma),[18] HPLC with diode array detection,[8,19-21] chemiluminescence detection,[22] amperometric detection,[23] and fluorescence detection,[24,25] and HPLC coupled with liquid-liquid micro-extraction,[26] and spectrofluorimetric methods.[27] However, the majority of them are either tedious, have complicated procedures, or employ costlier analytical instruments.

Interestingly, several noteworthy studies have reported the utilization of straightforward HPLC methods employing UV detection for quantifying VDF. However, it is worth highlighting that many of these methods have primarily focused on the quantification of VDF in dietary supplements, herbal matrices, and biological samples.[8,28,29] Furthermore, certain methods have exhibited longer retention times, such as 15.9 and 18 min, thus entailing increased solvent consumption and extended analysis duration, resulting in higher costs.[30,31] Notably, some approaches solely employing VHT have yielded elution times of 5.8 and 3.94 min, with poorly shaped peaks characterized by significant asymmetry and tailing effects, along with limited sensitivity in terms of linearity range.[32,33] These observations highlight the need for improved and distinguished analytical methodologies to address these limitations and enhance the accuracy and sensitivity of VHT quantification.

The goal of the method was to come up with a new analytical procedure that would address the problems with the methods that had been reported before and still accurately measure the amount of VHT in pharmaceutical formulations, aligning with the recommendations set forth by the International Conference on Harmonization (ICH). The anticipated method aimed to be simple, rapid, sensitive, accurate, and relatively inexpensive, besides being easy to use on a regular basis for analyzing pharmaceutical formulations. Additionally, this method offers a lower limit for identification and quantification, enhancing its applicability for analyzing formulations with minute doses, such as inhalations. By addressing existing challenges and providing improved analytical capabilities, this innovative approach holds significant potential for advancing the accurate measurement of VHT in pharmaceutical formulations.

MATERIALS AND METHODS

Reagents and chemicals

The Indian company Cadila Healthcare Ltd., in Thane, Maharashtra, gave a sample of vardenafil hydrochloride trihydrate. We bought analytical-grade Orthophosphoric Acid (OPA), sodium hydroxide, hydrochloric acid, peroxide, and HPLC purity-grade Acetonitrile (ACN) from Mumbai, India-based Merck Specialties Pvt. Ltd., for our analysis. We got 20 mg of vardenafil tablets made by Macleods Pharmaceuticals Ltd. from a local pharmacy. These tablets are named Varimax 20. For mobile phase preparation, we used freshly prepared water from LiChrosolv® and filtered it using 0.45 µm nylon 66 membrane filters, which we acquired from Sigma-Aldrich, India. Prior to the commencement of the study, all reagents utilized were meticulously prepared, subjected to comprehensive analysis, and diligently employed.

Instrumentation

For analysis, we used a HPLC instrument (Make: Agilent, Model: 1260 Infinity II) that was fitted with an auto injector (G7129A), a quaternary gradient pump (G1311B), a UV-Vis detector (G4212B DAD), and data processing software called OpenLab EZChrom (version 4.8). During the study, separation was accomplished with an Agilent Zorbax Bonus-RP column that was 250x4.6 mm and its internal particle diameter was 5 micrometers'. Also used were an analytical balance (Aczet CY224C), an ultrasonicator (Labman LMUC-6), and a Pall Pvt. Ltd., nylon 66 membrane filter of a 47 mm diameter and 0.45-micron pores.

Preparation of standard stock solution

A precisely measured quantity of 5 mg of standard VHT was combined with 10 mL of water in a 10 mL calibrated flask, yielding the standard stock solution. Subsequently, 5 mL of a diluent composed of 50% of 0.1% OPA and 50% ACN was added to the flask, ensuring complete dissolution of the drug through thorough mixing. The ultimate volume was brought to its desired level by adding the diluent to achieve a concentration of 500 µg mL^{-1}, followed by filtration using a membrane strainer of 0.45 microns. To obtain a final VHT concentration of 50 µg mL^{-1}, a quantity of 1 mL of this solution was mixed with diluents to achieve a final volume of 10 mL.

Preparation of Working Solution

To establish a comprehensive concentration series spanning from 0.0025-6 µg mL^{-1}, precise aliquots of standard stock solutions were judiciously diluted with 0.1% OPA and ACN in a harmonious 50:50 volumetric ratio. Dilution was meticulously carried out, ensuring that each working solution attained the targeted volume

Sample Solution Preparation (Marketed formulation)

To generate a representative sample solution, the marketed formulation of VHT, known as Varimax-20, was employed. Initially, the weight of 10 VHT tablets was measured, and then meticulously ground into a fine dust. Subsequently, an accurately measured quantity of this powder, containing 5 mg of VHT, was skillfully blended with 5 mL of a diluent within a volumetric container with a capacity of 10 mL. To ensure optimal dissolution, the resulting mixture underwent a sonication process for 15 min, thereby promoting thorough homogeneity. The volume was subsequently adjusted by incorporating the solvent mixture, thereby achieving a targeted concentration of 500 µg mL^{-1}. To ensure the removal of any particulate matter or impurities, this

solution was subjected to filtration through a porous membrane with a pore diameter of 0.45 µm. From the resulting filtered solution, an exactly measured volume of 1 mL was combined with diluents to attain a final concentration of 50 µg mL^{-1}.

Chromatographic Condition

The optimized composition for the mobile phase was observed to be 20% ACN and 80% OPA at a concentration of 0.1%. For the selected method, the stationary phase used was the Agilent Zorbax Bonus-RP C_{18} column, which measured 250x4.6 mm, and the internal particles measured 5 µm in size. Before use, the mobile phase was subjected to vacuum filtration using membrane filters made of nylon with an aperture size of 0.45 µm. The column was allowed to equilibrate with the solvent system for 30 min before injecting the solutions. The solvent system was subjected to the column and pumped at a flow rate of 1.0 mL min^{-1}. UV detection was carried out at 247 nm with a run time of 10 min and a sample loading volume of 10 µL and the column was kept at 30°C throughout the study.

Method Validation

The optimised chromatographic method has been verified in agreement with the 2005 ICH Q2 (R1) procedures. The validation process encompassed a comprehensive evaluation of key characteristics, including specificity, linearity, accuracy, precision, Detection Limit (LOD), and Quantitation Limit (LOQ). To assess precision, both intra-day reproducibility and intermediate reproducibility were assessed.

Specificity

Specificity means the ability to measure and separate an analyte of interest even when other expected components are also present. To ascertain whether the presence of other components had an impact on the developed method, a rigorous assessment of specificity was performed. This assessment entailed the analysis of blank samples, standard drugs, marketed formulations, and placebo solutions to recognize the method's ability to discriminate against interfering substances. Separate injections of blank samples, working standards, and drug products were precisely performed at a standardized concentration of 5 mg mL^{-1}. The resulting chromatograms were recorded under optimized conditions for measurement.

System Suitability

The consistent and reliable performance of the chromatographic system is paramount to ensuring accurate and precise analytical results. Following the protocols outlined in USP 24 and NF 19, we conducted an assessment to ascertain the system's suitability for analysis. A system suitability test on newly made standard solutions of VHT was carried out by injecting them into six replicates. The test encompassed a comprehensive evaluation of crucial parameters, including retention time, theoretical plates, and chromatographic asymmetry. By examining these parameters in the standard chromatogram, the performance of the system was thoroughly evaluated.

Linearity

A given analytical method can be deemed linear if it has the capacity to produce test outcomes that are linearly related to the amount of analyte present within a certain range in the sample. Examining a minimum of five strengths of an active compound served to demonstrate linearity. Simple linear regression was used to show that the method was linear, and the least squares method was used to evaluate it.[34] Several standard solutions of VHT were created, spanning ten different concentration levels ranging from 0.05% to 120% (equivalent to 0.0025 µg mL^{-1} to 6 µg mL^{-1} of VHT), and subsequently injected into the HPLC system. The resulting peak areas for all the peaks were precisely recorded, and a calibration curve was generated by graphing the peak area against the analyte concentration. The linear regression equation was applied to establish the gradient (a), intersection point (b), and coefficient of correlation (R^2).[35]

Accuracy

When performing the test more than once, the results' proximity to the pre-set reference value serves as a gauge of how accurate an analytical procedure is. Beer's range was tested at three different levels of concentration, between 80% and 120%.[36] By comparing the measured concentration to the concentration that was added, the percentage recoveries were calculated. The extent of substance recovery was determined by comparing the amount added with the amount measured. The evaluation metric utilized for assessing accuracy was the percent RSD.

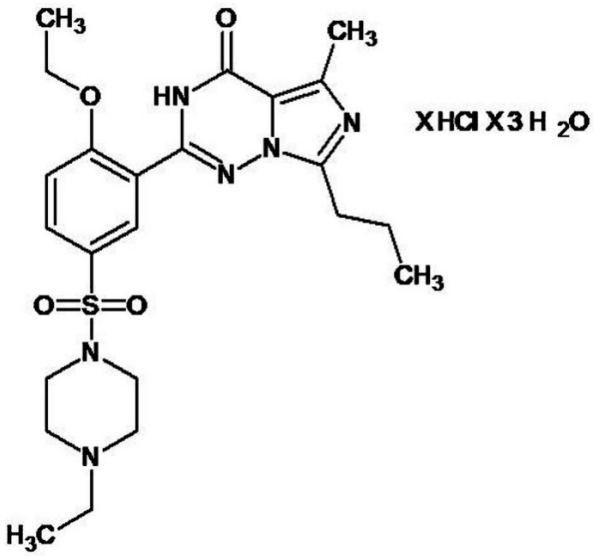

Figure 1: Structure of Vardenafil HCl trihydrate.

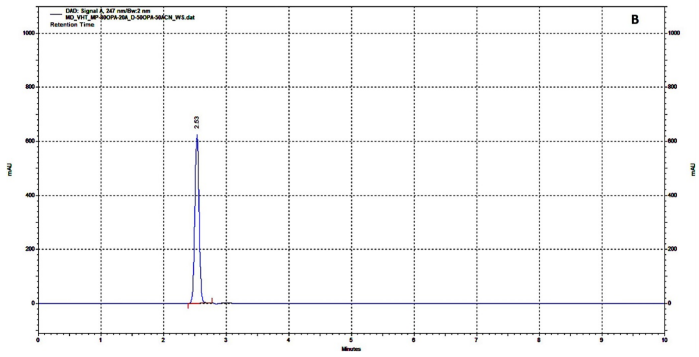

Figure 2: A typical HPLC chromatogram of VHT (standard).

Precision

It is based on the consistency of a group of measurements taken from several identical samples, all conducted under the same controlled conditions and exhibiting agreement among them. To evaluate the method's precision, both its instrumental precision and its ability to deliver consistent results within a day (intra-day) and between different days (inter-day) were determined. To assess precision, six distinct sample preparations of VHT were made from a single homogenous sample with a concentration of 5 µg mL^{-1}. By performing the analysis using different normal test conditions, including variations in analysts and equipment, an evaluation of intermediate precision was achieved. For each type of precision evaluated, the peak area was determined and represented as the average, standard deviation, and percent RSD.

Robustness

Robustness is a technique's ability to keep working normally even if one or more of its parameters are changed in a small but deliberate and significant manner. To judge the stability of the technique, a comprehensive study was conducted by performing minor modifications to key factors like the solvent composition in the mobile phase (with an increase in ACN from 70% to 90%), the speed of the eluent (ranging from 0.8 to 1.2 mL min^{-1}), and the temperature of the column (varying between 28°C and 32°C). The purpose of this study was to ascertain the impact of these modifications on crucial parameters, including retention times, theoretical plates, and tailing factors. By systematically analyzing the obtained data, the method's robustness and its ability to maintain consistent performance under varied conditions were assessed.

Sensitivity

The evaluation of a method's sensitivity is accomplished by computing the thresholds of detection and quantification. LOD is the smallest amount of analyte that can be reliably identified in a sample, while LOQ is the smallest amount of analyte that can be measured accurately and precisely in a sample. In accordance with the comprehensive guidelines outlined in the ICH, the LOD and LOQ were determined using robust statistical methods. By utilising the deviation of the response and the gradient of the corresponding standardisation curve, we were able to establish the LOD and LOQ values, thereby quantifying the method's sensitivity to detect and measure analyte in various samples.

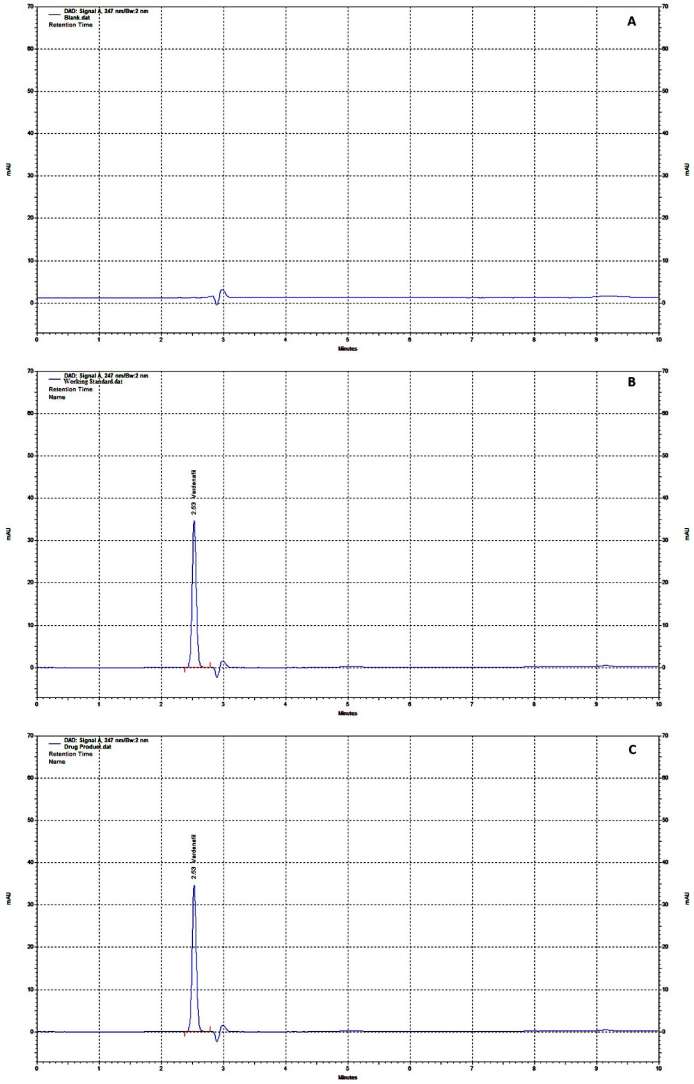

Figure 3: A typical Chromatogram showing the specificity of A) Blank B) Working Standard C) VHT Formulation.

Evaluation of the commercially available formulation

The retention times of the drug samples taken from the chromatograms stayed the same, indicating the absence of any noticeable interactions among the drug and added components present in the commercial formulations. Furthermore, the calculated percent RSD value provides a quantitative measure of the method's precision and shows its applicability for the prescribed testing of VHT in the preparation that was sold. These results not only reinforce the reliability and validity of the analytical approach but also affirm its practical applicability in the quality control and assessment of VHT-containing products on the market.

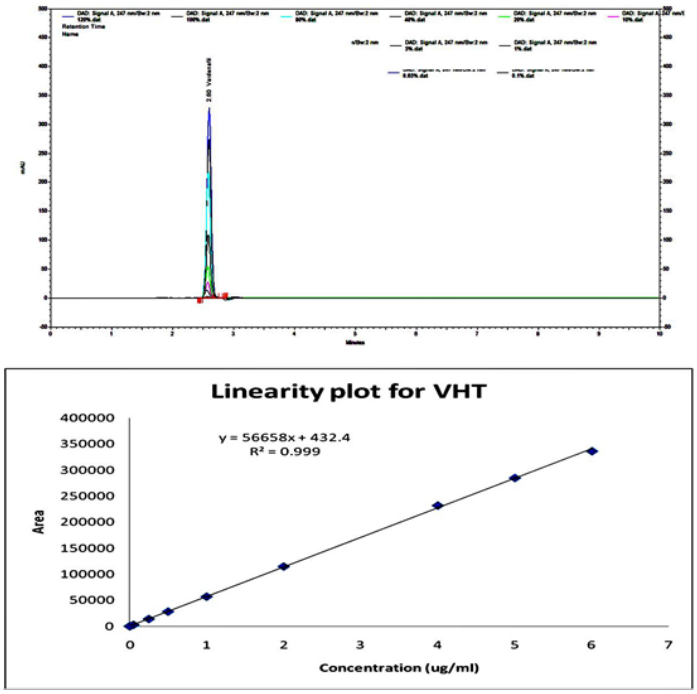

Figure 4: A typical Chromatogram showing the Linearity of VHT.

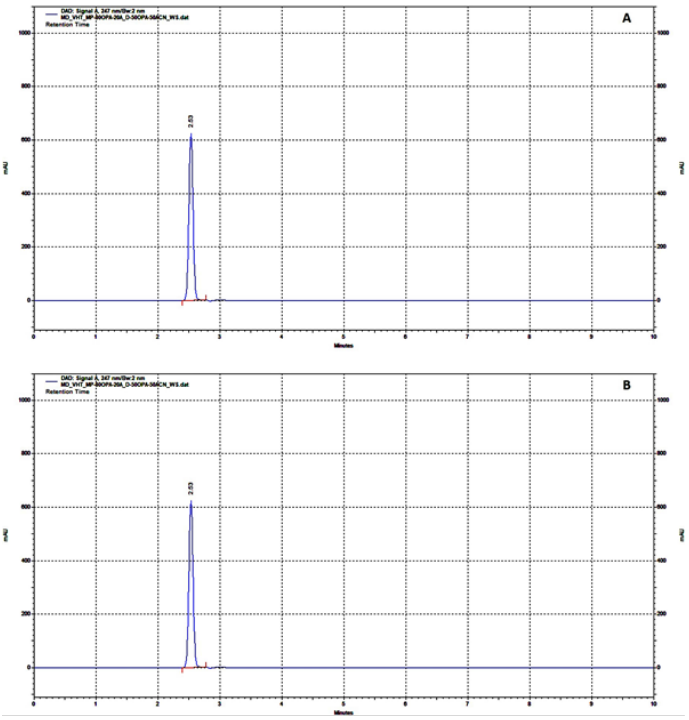

Figure 5: Accuracy Chromatogram of VHT at A) 80%, B) 100% and C) 120%.

Discussion

Numerous techniques have been utilized to measure VHT; however, a significant drawback lies in the lengthy retention times that range from 5.8 to 18 min,[7,18,31,32] along with their intricate and elaborate procedures, resulting in escalated analysis costs. Notably, the methods that have a shorter retention time of 3.94 min have exhibited unsatisfactory peak shapes and high asymmetry values.[33] To surmount these limitations and develop a more efficient method, diligent efforts were undertaken to ascertain an optimal mobile phase composition capable of achieving reduced retention times, minimizing tailing factors, and enhancing peak shape compared to the current methodologies. This pursuit aimed to establish a novel analytical paradigm that excels in terms of effectiveness, cost-efficiency, and analytical performance, setting the stage for advancements in VHT analysis.

Method Development and Optimization

The analysis employed the Agilent Zorbax Bonus-RP C18 column, featuring dimensions of 250 x 4.6 mm and the size of internal particles at 5 µm was used for all the trials. The pivotal trials revolved around the systematic modulation of the solvent system ratio, encompassing varying proportions of 0.1% OPA in water and ACN, namely 50:50, 60:40, 70:30, 80:20, and 90:10, while maintaining a fixed diluent of 50:50 of 0.1% OPA in water and ACN. These comprehensive trials were meticulously scrutinized gauging their impact on critical parameters such as Retention Time (RT), Theoretical Plates (TP), and asymmetry of VHT.

Increasing the proportion of ACN in the solvent system (50:50, 60:40, and 70:30) resulted in shorter retention times, indicating weaker interactions between VHT and the stationary phase and potentially leading to less effective separation. Concurrently, smaller peak areas and uneven peaks suggest possible peak tailing or compromised resolution. Conversely, employing lower ACN ratios (80:20, 90:10) yielded prolonged retention times, signifying stronger VHT-stationary phase interactions for better separation. Notably, reduced peak asymmetry denotes superior peak shape, a desirable attribute in chromatographic analyses.

One trial using 90:10 ratios exhibited an impressive TP of 18117 and a retention time (RT) of 3.04 min. However, its suboptimal peak shape and asymmetry value of 1.47 fell outside the acceptable asymmetry range (0.8 to 1.2) recommended by regulatory agencies such as the USP and ICH. As a result, this mobile phase ratio was excluded from further development and validation.

Instead, the mobile phase, comprising 80%, 0.1% OPA, and 20% ACN, emerged as the preferred choice. This selection yielded enhanced conditions for VHT analysis, as evidenced by a reduced RT of 2.53 min, a TP value of 6630, and an asymmetry measurement of 1.04, indicating a more efficient and effective separation method as shown in Figure 2. Furthermore, it is noteworthy that the chosen wavelength of detection (247 nm) exhibited consistent results across all tested conditions, implying a negligible impact on the chromatographic separation.

RESULTS AND DISCUSSION

The optimised method was evaluated in harmony with the US FDA and ICH guiding principles with other indexed journal articles for analytical testing procedure validation.[37-39]

Specificity

Comparing the chromatographic separation profiles of a blank sample, a sample obtained under optimal analytical conditions, and a standard solution allowed for a thorough evaluation of the method's specificity, as shown in Figure 3. The primary objective of this investigation was to ascertain the potential influence of the mobile phase, the placebo, and other excipients on the chromatographic system. Despite the presence of various excipients, the HPLC chromatogram exhibited clear differentiation of the VHT peaks. This unequivocally demonstrated the purity of the VHT compound and, more significantly, validated the specificity of the developed method by confirming its resilience to potential impurities or coexisting components. Consequently, the established analytical approach ensures accurate quantification and effective separation of the target analyte, even in the presence of complex sample matrices.

System Suitability Test

The comprehensive evaluation of system suitability parameters is presented in Table 1, showcasing the robustness and reliability of the developed analytical system.[40-45] The remarkably low values of percent RSD for these parameters serve as compelling evidence of the system's exceptional reliability and consistency. Specifically, the mean peak area of 330575±0.4622, indicates excellent reproducibility, underscoring the system's ability to consistently measure the analyte's response. The elution time of 2.53±0.2044 min highlights the system's precision. Moreover, the mean theoretical plates of 6788±0.7769 signify superior column efficiency and the system's capability to facilitate optimal separation of sample components. The asymmetry of the peak is within the acceptable range of 0.9 to 1.2, with a mean value of 1.06±0.9682, further exemplifies the system's ability to consistently generate symmetrical chromatographic peaks. It suggests that, the developed system is precise, reproducible, and appropriate for the intended analytical purpose, providing a good foundation for further sample analysis.

Linearity

The establishment of linearity is paramount for achieving accurate and reliable results across a broad range of analyte concentrations in HPLC method. In this investigation, the linearity of the method was carefully evaluated over the concentration array of 0.0025 to 6 µg mL^{-1}. Figure 4 showcases a representation of the strong linear relationship between concentration and response, as indicated by the substantial gradient (a) of 56658 in the calibration curve. Furthermore, a strong correlation between concentration and response is evident, with an R value of 0.9998. The determination coefficient (R^2) measuring 0.9997, elucidates that 99.97% of the response variation can be attributed to the concentration variation. In the tested concentration range, the established HPLC method was very accurate at measuring the analyte. The results reveal a strong linear relationship between concentration and response, supported by the high slope, correlation coefficient, and determination coefficient values as summarized in Table 2. Overall, the method is reliable and consistent, making it suitable for its intended analytical purpose.

Accuracy

Recovery studies were conducted using the standard addition method at three distinct concentration levels, spanning from 80% to 120%, to examine the accuracy of the HPLC method. Table 3 outlines the percentage recoveries of VHT, while Figure 5 illustrates the corresponding chromatograms. The average recovery percentage for all three concentrations was between 99.20% and 100.43%, indicating the high accuracy of the HPLC method. The observed RSD values were relatively low, ranging

Table 1: System suitability parameters.

Parameter	Observations* (Mean±%RSD)
Concentration (µg mL^{-1})	5.00
Peak Area	330575.80±0.4622
Retention time	2.53±0.2044
Theoretical Plates	6788±0.7769
Asymmetry	1.06±0.9682

*Average of six readings.

Table 2: Linearity values of VHT.

Parameters	VHT
Range (µg mL^{-1})	0.0025 to 6
Slope (a)	56658
Intercept (b)	432.4
Correlation coefficient (R)	0.9998
Determination coefficient (R^2)	0.9997

Table 3: Results of the accuracy of the HPLC method developed for VHT.

% Level	Spiked Conc. (µg mL^{-1})	Area	Amount Recovered (µg mL^{-1})	Average (%)	STDEV	RSD
80	3.988	265483.50	4.01	100.43	0.0372	0.04
100	4.985	330050.00	4.98	99.20	1.2639	1.27
120	5.982	397622.00	5.99	99.60	1.2297	1.23

from 0.04% to 1.27%, suggesting good precision for the method. Overall, these outcomes specify that the HPLC methodology is reliable for determining the VHT concentration in samples.

Precision

The correctness of the method was extensively assessed through a comprehensive evaluation, which included measurements at multiple levels: system, intra-day, and between-day. The exactness was thoroughly assessed through a detailed examination of six peak area measurements in the sample solution. Impressively, the results showed excellent precision, as shown by% RSD values of just 0.46, 0.47, and 1.08 for system, intra-day, and inter-day precision, respectively, as presented in Table 4. These notable outcomes clearly establish the method's reliability, reproducibility, and precision, surpassing the stringent criterion of maintaining a % RSD below the esteemed threshold of 2%.

Robustness

During the laborious investigation of robustness, it was recognized that even minor deviations in the chromatographic conditions had negligible influence on the analytical parameters, as documented in Table 5. Particularly, variations in the elution rate of the solvent, ranging from 0.8 to 1.2 mL min^{-1}, and the percentage of ACN, transitioning from 70% to 90%, caused only marginal alterations in retention time, asymmetry, and theoretical plate count, which lacked statistical significance. Moreover, alterations in column temperature from 28°C to 32°C exhibited no observable impact on the chromatographic parameters, affirming the method's rigidity to temperature changes. These findings not only ascertain the robustness of the method but also reveal optimal chromatographic conditions, promoting enhanced separation, retention, and efficiency without compromising reliability.

Sensitivity

The employed test method within this study exhibits exceptional proficiency in identifying and computing the analyte, even at minute concentrations. Table 6 reveals noteworthy values for the LOD and LOQ, measuring 56.91 ng mL^{-1} and 172.44 ng mL^{-1}, respectively. The lower values of LOD and LOQ indicate enhanced sensitivity of the method. This experimental value underscores the significant sensitivity of the test method towards the target analyte, enabling the identification and accurate quantification of nanogram-level quantities of VHT within the sample with exceptional accuracy.

Formulation

The assay outcomes obtained from analyzing the VHT drug within the commercial formulation showed a measured concentration of 49.85 µg mL^{-1}, corresponding to an impressive 99.70% of the labelled quantity (5 mg). Evidenced by a low percent RSD value of 0.4578, the method demonstrated high precision. Consequently,

Table 4: Precision values of VHT by the proposed method.

Observations* (Mean±%RSD)			
Parameter	**System Precision (Repeatability)**	**Method Precision (Intra-day)**	**Intermediate Precision (Inter-day)**
Peak Area	330575.80±0.4622	295673.00±0.47	285456.00±1.08
Retention time	2.53±0.2044	2.60±0.1989	2.60±0.1571
Theoretical Plates	6788±0.7769	6102±0.4717	5891±1.0853
Asymmetry	1.05±1.72	1.06±1.2520	1.05±1.7620

*Average of six/nine readings.

Table 5: Robustness parameters of VHT.

Parameters	Value	Retention Time (Mean±RSD)	Asymmetry (Mean±RSD)	Theoretical Plates (Mean±RSD)
Flow rate (mL min^{-1})	0.8	2.14±0.3421	1.08±1.6564	4958±1.5454
	1	2.60±0.6749	1.00±1.6565	6018±1.6564
	1.2	3.22±1.2436	1.49±0.8594	7206±0.9874
Mobile phase (% Acetonitrile)	70	1.83±0.3453	1.05±1.6904	7450±1.3434
	80	2.53±1.5367	1.04±0.9872	6630±0.9087
	90	3.04±1.8865	1.47±1.5646	18117±1.4535
Column Temperature (°C)	28	2.60±0.9898	1.00±1.8675	6317±1.0483
	30	2.60±0.5739	1.00±1.7940	6018±1.4535
	32	2.60±0.7496	1.00±1.0938	6118±1.8676

Table 6: Sensitivity data of VHT.

Parameter	Values (ng mL^{-1})
Detection limit (LOD)	56.91
Quantitation limit (LOQ)	172.44

Table 7: Assay data of marketed formulation.

Drug	Amount labelled	Amount found* µg mL^{-1}	% Assay	% RSD
VHT	5 mg	49.85	99.70	0.4578

Table 8: Figures of Merits.

Authors	Column, Mobile Phase, Flow rate	RT (min)	Linearity µg mL^{-1}	LOD µg mL^{-1}	Recovery (%)	Applications	References
Patel B *et al.* 2017	C18 column, Isocratic, HPLC-UV (5:51:44) THF: Methanol: Phosphate buffer, pH-6.5 with 0.1% TEA.	18	1-6	0.0319	99.72-99.96	Pharmaceutical Formulation and food adulterants	31
Subba Rao DV *et al.* 2008	C18 column, Gradient, HPLC-UV Phase A: (80:20 v/v) 10 mM H_4NaO_5P buffer, 1 mL of TEA and ACN, pH 7.5 with OPA, Phase B: (60:40 v/v) ACN and water, 1.0 mL/min.	15.9	50-150	0.005	99.40- 101.2	Production Samples	30
Gratz SR *et al.* 2004	C4 Column, LC-ESI-MS (68:28:4) (H20: acetonitrile: buffer), pH 4.5, 1.5 mL/min.	10.80	--	--	Vardenafil not found	Dietary Supplements and herbals	7
Carlucci G *et al.* 2009	C18 column, Gradient, HPLC-UV (30:70 v/v) ACN-70 mM potassium dihydrogen phosphate, pH 4.5 with OPA, 1.0 mL/min.	6.4	0.01-1.5	0.005	98.0- 99.6	Human Plasma	18
Aboul-Enein HY *et al.* 2005	C18 column, HPLC-UV 20:80 (v/v) acetonitrile-10 mM phos.buffer, pH 3.0, 1 mL/min.	5.8	10-1000	0.10	103.0-107.0	Pharmaceutical formulations	32
Manisha G *et al.* 2013	C8 column, HPLC-PDA (60:40, v/v) pot. dihydrogen phos.buffer pH 4.5 and ACN, 1.0 mL/min.	5.4	50-250	0.0125	>98.0	Pharmaceutical formulations	20
Tero-Vescan A *et al.* 2014	C8 column, Gradient, HPLC-UV (A=0.1% formic acid in water) and (B =0.1% formic acid in ACN), 1.0 mL/min.	4.67	--	--	--	Dietary supplements	28
Rishitha C *et al.* 2016	C18 column, HPLC-PDA methanol: water (80:20), 1.0 mL/min.	3.94	40-140	0.01	99.80-101.50	Bulk drug	33

Authors	Column, Mobile Phase, Flow rate	RT (min)	Linearity µg mL^{-1}	LOD µg mL^{-1}	Recovery (%)	Applications	References
Nickum EA *et al.* 2015	C8 column, Gradient, HPLC-UV Solvent A: 0.1% TFA in H2O and Solvent B: 0.1% TFA in CH_3CN, 1.0 mL/min.	3- 4	1-10 and 250-400	0.2	102.00	Dietary supplements	29
Kumar KK *et al.* 2012	C18 column, UPLC-UV 20 mM Ammonium bicarbonate buffer pH 5.0 with OPA, acetonitrile.	2.9	250- 1000	0.017	99.8- 100.8	Production samples	16
Aboul-Enein HY *et al.* 2005	Monolithic column, HPLC-UV 30:70 (v/v) ACN-10 mM phos. buffer, pH 3.0, 2 mL/min.	1.7	10-1000	0.11	103.0-107.0	Pharmaceutical formulations	32
Patel MN *et al.* 2021	C18 and C8 columns, Dual Gradient, HPLC-PDA A: Water: CAN (80:20), B: Water: CAN (40:60).	18.84	10- 35	0.35	98.8- 101.5	Bulk drug	21
Our Method	C18 column, Isocratic, HPLC-UV 80:20 (v/v) 0.1% OPA and ACN, 1.0 mL/min.	2.53	0.0025-6	0.0569	99.88-100.43	Bulk drug, Pharm. formula	Proposed Work

these findings clearly establish the method's capacity to provide accurate and reliable estimations of VHT content within market-available formulations, as represented in Table 7.

Figure of Merits

The quantification of VHT remains a topic with limited literature coverage, as evidenced by the scarcity of reported analytical methods. However, there are several ways to measure vardenafil and vardenafil hydrochloride in pharmaceutical dosage forms, herbal matrices, dietary supplements, biological samples, etc.[18,29,32]

Several pioneering studies have contributed to the development of distinct HPLC methods for the precise determination of vardenafil and its salts. Noteworthy among them are Subba Rao DV *et al.*, 2008; Patel Bhargav *et al.*, 2017; Rishita C *et al.*, 2016; Gratz SR *et al.*, 2004; Carlucci G *et al.*, 2009; Aboul Enein HY *et al.*, 2005; Manisha G *et al.*, 2013; Tero-Vescan A *et al.*, 2014; Nickum EA *et al.*, 2015. Remarkably, all of these methods exhibited significant sensitivity with better limits of detection. Though, it is worth noting that their retention times varied between 4 to 18 min, as presented in Table 8. Although, their sensitivity is commendable, the extended retention times leads to increased solvent consumption and time investment, potentially resulting in higher costs. In contrast, our method exhibits noteworthy advantages in terms of speed and cost-effectiveness. Particularly, our approach showcases a significantly reduced retention time of merely 2.53 min, rendering it markedly faster. Moreover, it stands as a more economical alternative, requiring less time and solvent consumption compared to the aforementioned techniques.

While Kumar KK *et al.* (2012) and Aboul Enein HY *et al.* (2005) achieved RT that closely resembled ours, their utilization of UPLC instruments and monolithic columns introduced higher analysis costs. Moreover, the methodologies employed by Subba Rao, DV, *et al.* (2008), Carlucci G *et al.* (2009), Tero-Vescan A *et al.* (2014), and Nickum EA *et al.* (2015) involve intricate gradient elution processes, in contrast to our method's simplicity through isocratic elution. Our approach stands out for its streamlined nature, requiring no complex steps in sample preparation or mobile phase composition. Furthermore, our method's superiority lies in its cost-effectiveness, which is attributed to the higher proportion of water and lower proportion of organic solvent utilized.

The HPLC chromatograms presented by Aboul Enein HY *et al.*, 2005, and Rishita C *et al.*, 2016, revealed the presence of poorly shaped peaks, visible tailing effects, and considerable asymmetry. In contrast, our method has successfully achieved sharper peaks with reduced tailing effects compared to both of these approaches.

Furthermore, the methodologies developed by Gratz SR *et al.* (2004) and Tero-Vescan A *et al.* (2014) lack comprehensive information regarding crucial aspects such as validation parameters and the recovery of VDF in analysed samples. These

elements play a vital role in the method development process. In contrast, our method's validation results demonstrate its accuracy, with a mean recovery ranging from 99.20% to 100.43%. Additionally, our method exhibits more sensitivity compared to other approaches, covering a linear range of 0.0025-6 g mL^{-1}.

Likewise, it is worth mentioning that the analysis of VDF in the studies conducted by Manisha G. *et al.* (2013), Tero-Vescan A. *et al.* (2014), and Nickum E.A. *et al.* (2015) utilized C8 columns. However, it is important to highlight that C18 columns possess higher hydrophobicity in comparison to C8 columns. This characteristic makes C18 columns a superior choice for the separation of polar compounds, such as vardenafil.

CONCLUSION

Based on the data obtained in this study, we have successfully achieved our research goal, which was to develop a rapid, accurate, and efficient method for detecting and quantifying Vardenafil Hydrochloride Trihydrate (VHT) in bulk drugs and finished products, with the lowest possible level of detection and measurement. Our HPLC method, utilizing C18 stationary phase and an isocratic eluent comprising of 0.1% OPA (80%) and 20% ACN proved to be highly effective. This method demonstrated several advantages over previously reported techniques. It displayed a remarkably short retention time of only 2.53 min, along with a good peak shape and minimal tailing effects.

In addition to its speed and efficiency, our approach offers cost-effectiveness and simplicity by eliminating the need for complex sample preparation or a gradient mobile phase.

This study confirmed the method's accuracy, precision, linearity, and specificity, with average recoveries, ranging from 99.20% to 100.43% highlight its reliable performance. Moreover, the method displayed greater sensitivity within the linearity range of 0.0025-6 µg mL^{-1}, surpassing the capabilities of alternative techniques. The technique's thresholds for recognition and quantification thresholds were 0.0569 ng mL^{-1} and 0.1724 ng mL^{-1}, respectively. This high sensitivity and precision enable the method to detect even nanogram amounts of VHT with utmost accuracy. Our developed method not only surpasses existing techniques in terms of speed, cost-effectiveness, and precision but also offers the capability to detect even nanogram levels of VHT with unparalleled accuracy, making it a valuable tool for pharmaceutical quality control and analysis.

In the next phase of our research, we will do thorough studies of forced degradation to come up with a stability-indicating method to measure the exact amount of VHT. This approach will provide insights into VHT's behaviour under various stress conditions and facilitate the identification of degradation products. Additionally, we aim to adapt this method for quality control analysis of pharmaceutical dosage forms in nanogram quantities, specifically targeting inhalational forms. This work promises to enhance pharmaceutical precision and patient safety, advancing the field of pharmaceutical analysis and quality control.

ACKNOWLEDGEMENT

Principal of Appasaheb Birnale College of Pharmacy in Sangli (Maharashtra), is acknowledged for providing the essential facilities required for conducting the research. Additionally, the authors are grateful to Cadila Healthcare Ltd., Thane, for providing the vardenafil hydrochloride trihydrate sample used in the study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ACN: Acetonitrile; **GC-MS:** Gas Chromatography-Mass Spectrometry; **HPLC-UV:** High Performance Liquid Chromatography-Ultraviolet detection; **ICH:** International Conference on Harmonization; **ESI-MS:** Electrospray Ionization-Mass Spectrometry; **MS/MS:** Tandem mass spectrometry; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **min:** Minute; **mL:** Millilitre; **ng mL^{-1}:** Nanograms per millilitre; **OPA:** Orthophosphoric acid; **RT:** Retention time; **TP:** Theoretical plate number; **UPLC-DAD:** Ultra-Performance Liquid Chromatography-Diode Array Detection; **TOF:** Time of Flight mass spectrometry; **UV/VIS:** Ultraviolet/Visible Spectroscopy; **VDF:** Vardenafil; **VHT:** Vardenafil hydrochloride trihydrate; **µg mL^{-1}:** Microgram per millilitre; **µg:** Microgram; **µm:** Micrometer; **% RSD:** Percentage Relative Standard Deviation.

SUMMARY

This research addresses significant drawbacks in reported analytical methods and aims to overcome issues like time-consuming procedures, limited sensitivity, and high costs. The primary goal was to devise an innovative approach for identifying and measuring VHT in bulk drugs and finished products with unparalleled levels of precision and detection. The methodology used a C18 column and a solvent combination of 0.1% OPA and ACN (80:20). The process proved to be exceptionally sensitive, capable of identifying concentrations as low as 56.91 ng mL^{-1}. Our RP-HPLC method offers a distinctive combination of speed, accuracy, and sensitivity, outperforming established techniques. Because it can precisely measure nanogram levels of Vardenafil Hydrochloride Trihydrate (VHT), it is an essential tool for testing and controlling the quality of pharmaceuticals.

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Cite this article: Gadekar GR, Doijad RC, Jadhav NR, Bhinge SD. A Validated Simple, Rapid and Accurate RP-HPLC Approach for Measuring Vardenafil Hydrochloride Trihydrate in Bulk Drugs and Medicinal Formulations. Indian J of Pharmaceutical Education and Research. 2024;58(2s):s532-s542.