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DEVELOPMENT OF NOVEL MICROWELL-PLATE SPECTROPHOTOMETRIC ASSAY FOR DETERMINATION OF VARENICLINE VIA ITS REACTION WITH CYCLOHEXA-3,5-DIENE-1,2-DIONE

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This study describes, for the first, the spectrophotometric investigation for the condensation reaction between varenicline (VRC) and cyclohexa-3,5-diene-1,2-dione (CHDD). The reaction gave a violet-colored product exhibiting maximum absorption peak (λ_{max}) at 540 nm. The variables affecting the reaction were carefully investigated and the optimum conditions were established. The stoichiometry of the reaction was determined, and the reaction pathway was postulated. This color-developing reaction was employed in the development of microwell plate assay for VRC. In this assay, the reaction was carried out in 96-microwell plate and the absorbance of the colored-product was measured by microwell plate absorbance reader. Under the optimized reaction conditions, Beer's law correlating the absorbance with VRC concentration was obeyed in the range of 5 - 100 µg/mL with good correlation coefficient (0.9986). The limits of detection and quantification were 2.29 and 6.95 µg/mL, respectively. The assay showed high precision as the values of relative standard deviations (RSD) did not exceed 2%. No interference was observed from the excipients that are present in VRC-containing tablets. The proposed assay was applied successfully for the determination of VRC in its pharmaceutical tablets with good accuracy and precisions; the label claim percentages were 98.80 ± 1.30%. The results were compared favorably with those of a reference pre-validated method. The proposed assay is practical and valuable in terms of its routine application in determination of VRC in in its bulk and tablets in pharmaceutical quality control laboratories.

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1. Introduction

Varenicline (VRC) is a novel agent that is a centrally acting as a highly selective partial agonist for the nicotinic acetylcholine receptor [1]. VRC has mixed agonistic-antagonistic properties, thus it has the therapeutic benefit of relieving the symptoms of nicotine withdrawal and cigarette craving during abstinence while blocking the reinforcing effect of nicotine in those who lapse [2-4]. VRC tartrate has been approved by the USA-FDA as an aid to smoking cessation [5]. The approved regime of VRC is 1 mg for 12 weeks, starting with a one-week titration period [6].

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The effective and safe therapy with VRC is basically depending on the quality of its pharmaceutical preparations (tablets), and assessing its concentrations in tablets for the purposes of quality control. As well, the therapeutic benefits profile of VRC is anticipated to encourage the development of new pharmaceutical preparations for VRC. As a consequence, there is an increasing demand for a proper analytical method for determination of VRC in its bulk drug and finished pharmaceutical formulations. VRC has not yet been officially described in any pharmacopoeia. In literature, few methods have been found describing the quality control of VRC [7-11]. However, these methods involved complex and expensive instrumentation such as ultra-pressure liquid chromatography (UPLC) [8], LC-tandem spectrometry [9] and capillary electrophoresis [10]. Furthermore, the analytical throughputs of these methods were not high, thus their applications in routine determination of VRC is limited. For these reasons, the development of an alternative simpler methodology for determination of VRC was very important.

Spectrophotometry has a considerable importance in drug analysis, and the spectrophotometric methods are extensively applied in pharmaceutical analysis [12]. The importance of these methods in the field of pharmaceutical analysis has greatly increased, due to the fact that these methods can be very readily automated. Automated analyzers equipped with photometric detection are widey used for the serial analysis of pharmaceutical preparations, especially for studying the contents uniformity and dissolution characteristics of the solid dosage forms [12]. Unfortunately, no spectrophotometric method was reported for determination of VRC. The present study described the development of a novel microwell-plate spectrophotometric assay for determination of VRC. The method was based on the reaction of VRC with cyclohexa-3,5-diene-1,2-dione (CHDD) to produce a violet-colored product. The reaction was carried out in 96-microwell assay plate and the absorbance was measured by microwell absorbance plate reader. The proposed assay provided a high throughput methodology that can facilitate the processing of large number of samples in a relatively short time in pharmaceutical quality control laboratories.

2. Experimental

2.1. Apparatus

Microplate/cuvette reader (Spectramax M5, Molecular Devices, California, USA) was used for all the measurements in 96-microwell plates. Double beam V-530 (JASCO Ltd., Kyoto, Japan) ultraviolet-visible spectrophotometer with matched 1-cm quartz cells was used for all the spectrophotometric measurements. 96-Microwell plates were a product of Corning/Costar Inc. (Cambridge, USA). Finnpipette adjustable 8-channel-pipette was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Reagents and material

Varenicline tartarate standard with claimed purity of 99.6% was purchased from Weihua Pharma Co. Ltd. (Zhejiang, China). Cyclohexa-3,5-diene-1,2-dione (CHDD) reagent solution (0.3%, w/v) was prepared by oxidation of catechol with silver oxide according to the previously described procedures [13] with modification; methanol was used as a solvent instead of acetone to avoid the harmful effect of acetone of the microwells of the assay plate. Champix® tablets (Pfizer Inc, New York, USA), labeled to contain 1 mg VRC per tablet was obtained from the local market. Double distilled water was obtained through WSC-85 water purification system (Hamilton Laboratory Glass Ltd., Kent, USA) and used throughout the work. All solvents and materials used throughout this study were of analytical grade.

2.3. Preparation of VRC standard solution

An accurately weighed amount (20 mg) of VRC was quantitatively transferred into a 10-mL calibrated flask, dissolved in 5 mL methanol, completed to volume with the same solvent to obtain a stock solution of 2 mg/mL. The stock solution was found to be stable for at least two weeks when kept in a refrigerator. The stock solution was further diluted with methanol to obtain working solutions in the range of 10 - 200 μg/mL.

2.4. Preparation of tablets sample solution

Ten Champix® tablets were weighed and crushed to a fine powder. An accurately weighed quantity of the tablet powder equivalent to 20 mg of VRC was transferred into a 10-mL calibrated flask, and dissolved in about 5 mL of methanol. The contents of the flask were swirled, sonicated for 20 min, and then completed to volume with the same solvent. The contents were mixed well and centrifuged at 6000 rpm for 10 min; the supernatant was separated and filtered, the first portion of the filtrate was rejected. The filtered solution was diluted quantitatively with methanol to obtain working solutions in the range of 10 - 200 μg/mL.

2.5. General recommended procedures

An accurately measured volume (100 μL) of VRC solution containing 10 - 200 μg/mL was transferred into wells of 96-microwell plate. A 100 μl of CHDD solution (0.3%, w/v) was added. The reaction solution was allowed to proceed for 15 min at room temperature (25 ± 2 °C) and the resulting color was measured at 540 nm.

2.6. Determination of stoichiometric ratio by Job's method

The Job's method [14] of continuous variation was employed. Master equimolar (1×10^{-3} mol/L) solutions of VRC and CHDD were prepared. Series of 200 μL portions of the master solutions of VRC and CHDD were made up comprising different complementary proportions (0:200, 10:190, . . . , 190:10, 200:0, inclusive) in microwells. The solutions were further manipulated as described under the general recommended procedures.

3. Results and discussion

3.1. Strategy and design of the study

The chemical structure of VRC and its absorption spectrum are given in Fig. 1. It is obvious from the spectrum that VRC exhibits two maximum absorption peaks (λ_{max}) at 237 and 322 nm. Because of the blue shifted λ_{max} of VRC, its determination in the pharmaceutical formulations (tablets) based on the direct measurement of its absorption for ultraviolet light is susceptible to potential interferences from the co-extracted tablet excipients. Therefore, derivatization of VRC to a more red-shifted light-absoping derivative was necessary. VRC contains a secondary amino group for which many chromogenic reagents could be used for color-producing reactions. These reactions include formation of colored charge-transfer complex with electron acceptor [15], formation of ion-pair associates with pairing reagents [16-18], and formation of condensation product with a chromogenic reagent [19]. However, these methods are usually associated with some major drawbacks such as laborious multiple extraction steps in the analysis by ion-pair based methods [16-19], or in preparation of the free base of the drug prior to the analysis by charge-transfer-based methods [15], and long reaction time, thus the procedure is time-consuming [20].

Cyclohexa-3,5-diene-1,2-dione (CHDD) has 2 *ortho*-carbonyl groups that can exhibit substitution reaction with secondary amino group-containing molecules such as VRC. Therefore, the present study was devoted to investigate the reaction of CHDD with VRC for the purpose of its evaluation as color-developing reagent for VRC and its employment in the development of simple spectrophotometric method for determination of VRC.

The proposed assay was designed to employ 96-microwell assay plate as the reaction between VRC and CHDD was carried out in microwells of the assay plate (200-μl total reaction volume). The solutions were dispensed by 8-channel pipette, and the absorbance of the colored-reaction product was measured by 96-microwell-plate absorbance reader. The 96-microwell design of the proposed assay was considered based on the previous success of Darwish *et al.* [21,22] in the employment of this methodology for the high-throughput analysis of some other pharmaceuticals, and reducing the organic solvents-involving analytical procedures. The absorption spectra and the involved studies will be described in the following paragraphs.

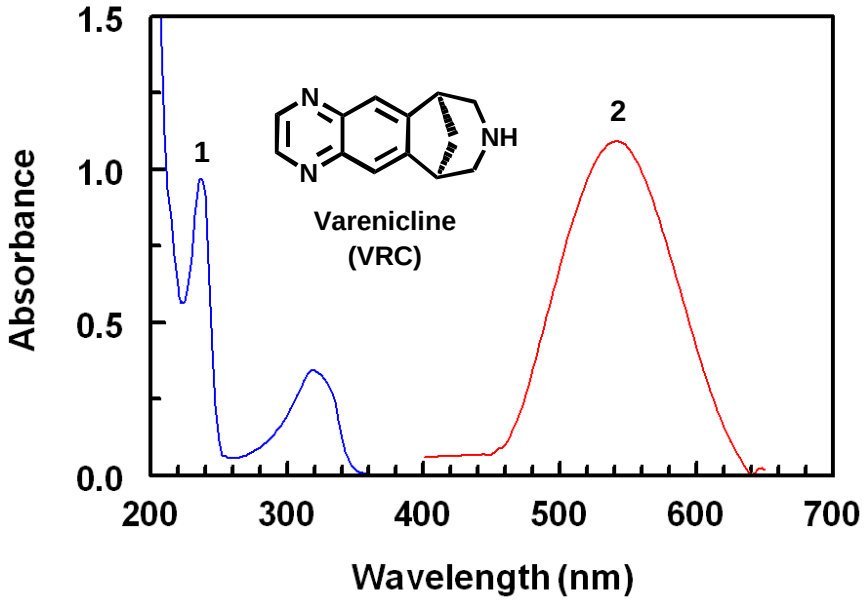

Fig. 1. Chemical Structure of VRC, absorption spectra of aqueous VRC solution (10 µg/mL) against water (1) and the reaction product of VRC (45 µg/mL) with CHDD solution (0.3%, w/v) against reagent blank (2).

3.2. Absorption Spectra

The reaction between VRC and CHDD was performed, and the absorption spectrum of the reaction product was recorded against reagent blank. The product was violet-colored exhibiting λ_{max} at 540 nm (Fig. 1). Obviously, the λ_{max} of VRC-CHDD derivative was significantly red-shifted from the underivatized VRC. This shift enables the measurements in the visible region and eliminates the potential interference from tablet excipients.

3.3. Optimization of Reaction Conditions

The optimization of experimental conditions affecting the reaction in the 96-microwell format was investigated by altering each reaction variable in a turn while keeping the others constant. All the measurements were carried out by the plate reader at 540 nm.

3.3.1. Effect of CHDD Concentration

In the chemical derivatization-based spectrophotometric analysis, the maximum conversion of the analyte into absorbing specie depends on the amount of the reagent available in the reaction solution. The effect of CHDD concentration on its reaction with VRC was studied using varying CHDD concentrations (0.05–0.5%, w/v). The results (Fig. 2) indicated that 100 µl of 0.3 (%, w/v) was the optimum CHDD concentration, as this concentration gave the highest absorbances. As shown in Fig 2, when the VRC molar concentration was twofold the CHDD concentration there was a total derivatization of VRC, indicating that one mole of CHDD reagent reacts with two moles of VRC.

3.3.2. Effect of Temperature and Time

The effect of temperature on the reaction was studied by carrying out the reaction at room temperature (25 ± 2 °C) and at varying elevated temperatures (25–60 °C). Elevated temperature was found to enhance the reaction rate; however, it stimulated the evaporation of the solvent as well. This effect resulted in poor-precise readings as indicated by the increase in the relative standard deviation (RSD) values (Fig. 3); therefore, further experiments were carried out at room temperature.

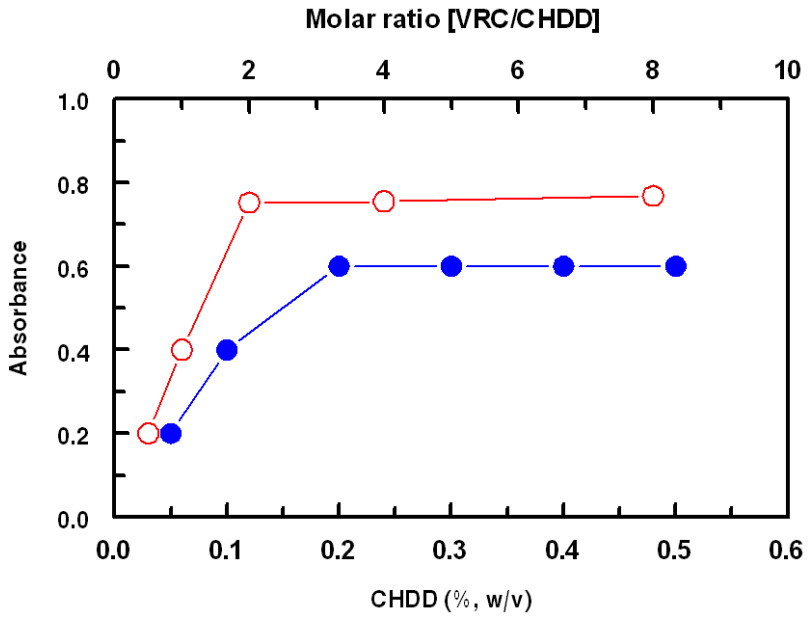

Fig. 2. Effect of CHDD concentration (%, w/v; ●) and molar ratio of VRC:CHDD (○) on the reaction of VRC (45 µg/mL) with CHDD.

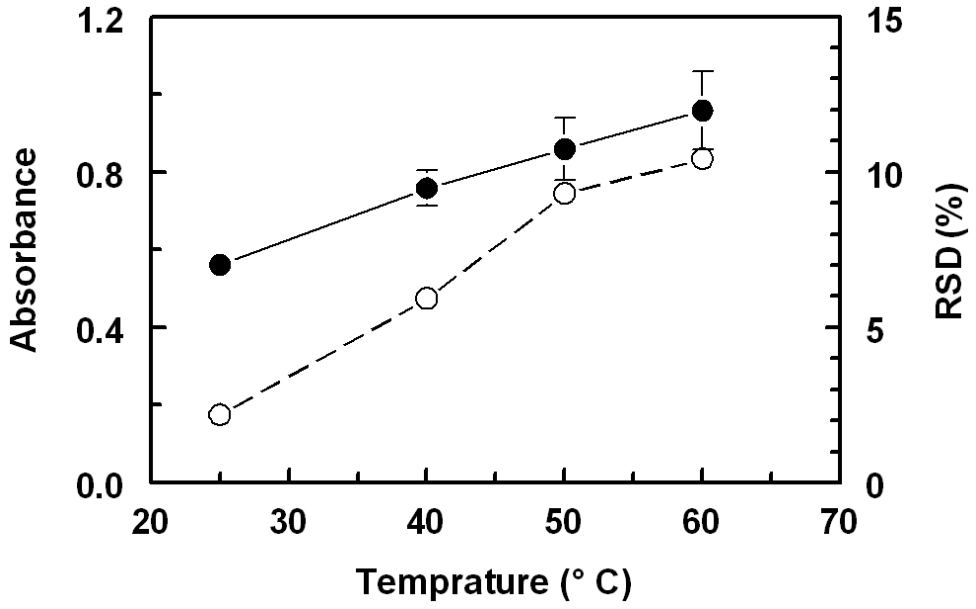

Fig. 3. Effect of temperature (●) on the reaction of VRC (45 µg/mL) with CHDD (0.3%, w/v), and precision profile of the readings (○).

In order to determine the optimum time that is required for completion the reaction, it was allowed to proceed at room temperature for varying periods of time. It was found that the reaction was dependent on time, and it went to completion after 10 min (Fig. 4); however, for higher precision readings, the reaction was allowed to proceed for 15 min in all the subsequent experiments.

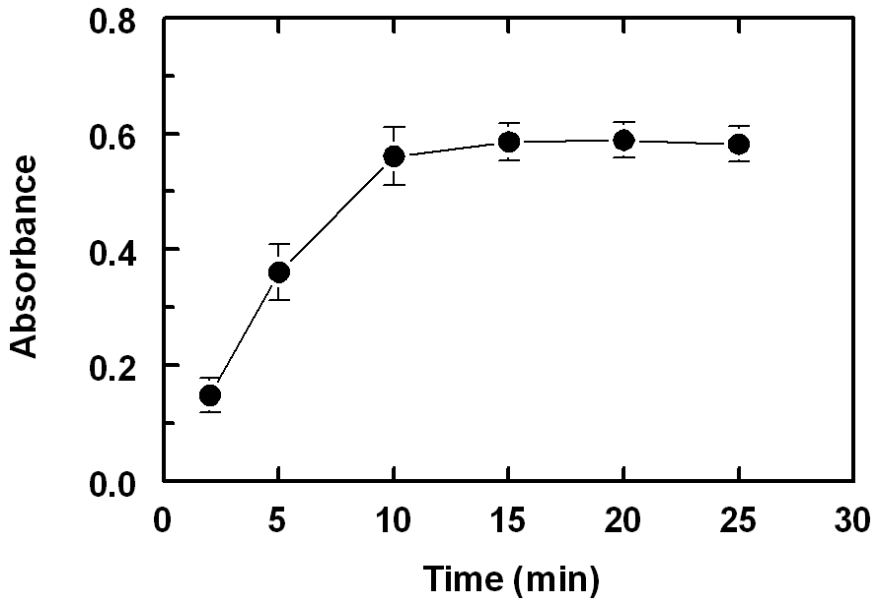

Fig. 4. Effect of time on the reaction of VRC (45 μg/mL) with CHDD (0.3%, w/v).

3.3.3. Effect of Solvent

In order to select the most appropriate solvent for solubilization of the CHDD and carrying out the reaction with VRC, different solvents (water, acetone, methanol, ethanol, n-propanol, isopropanol, and propane-2-one) were tested. Acetone was found to be negatively affecting the transparency of the microwells of the assay plate; therefore it was ruled out from further experiments. Formation of unclear cloudy reaction solution was obtained when water was used as a solvent for VRC. This was attributed to probably the precipitated of the CHDD in this aqueous-organic (1:1) solution. The highest readings were obtained when methanol was used; therefore, it was used in all the subsequent experiments.

3.3.4. Stability of the Chromogen

The effect of time on the stability of the VRC-CHDD chromogen was studied by following the absorption intensity of the reaction solution at different time intervals. It was found that the absorbance of the chromogen remains stable for at least 1 h. This allowed comfortable analytical processing of large batches of samples. This gives a high throughput property to the proposed method when applied for analysis of large number of samples in quality control.

A summary for the optimum conditions for the reaction between VRC and CHDD is given in Table 1.

Table 1. Summary for the optimization of variables affecting the reaction of VRC with CHDD reagent employed in the development of the proposed microwell-plate spectrophotometric assay for VRC.

Variable	Studied range	Optimum
CHDD concentration (%, w/v)	0.05 – 0.5	0.3
Temperature (°C)	25 – 60	25
Time (min)	2 – 25	15
Solvent	Different[a]	methanol
Chromogen stability time (min)	5 – 90	60
Measuring wavelength (nm)	400 – 600	540

[a] Solvents were: water, acetone, methanol, ethanol, n-propanol, isopropanol, and propane-2-one.

3.4. Stoichiometry and Mechanism of the Reaction

Under the optimum conditions (Table 1), the stoichiometry of the reaction between VRC and CHDD was investigated by Job's method [14]. The results indicated that the VRC:CHDD ratio was 2:1 (Fig. 5). This indicated that two moles of VRC interacted with one mole of CHDD and confirmed the assumption rose before when the effect of CHDD concentration was studied (Fig. 2). Considering the presence of only one–NH group in VRC structure, the reaction was postulated to proceed as shown in Fig. 6.

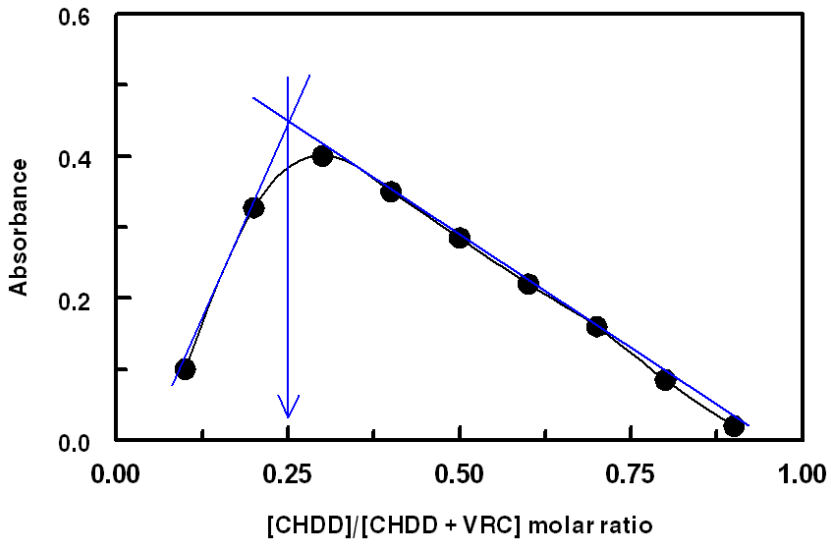

Fig. 5. Job's plot for the reaction of VRC with CHDD.

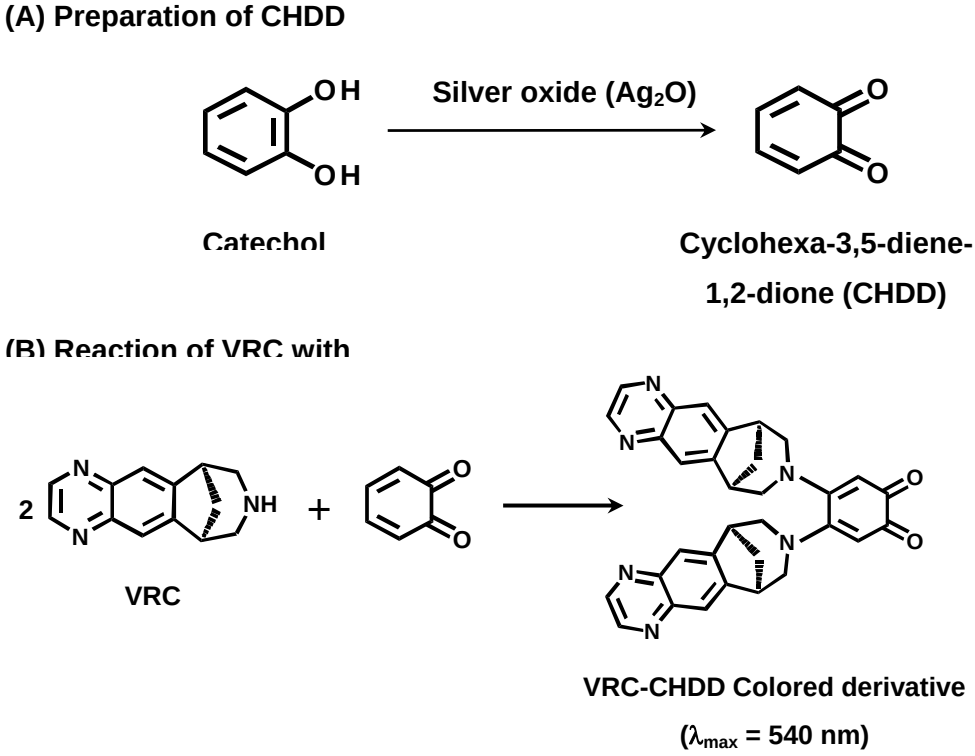

Fig. 6. Scheme for the preparation of CHDD (A) and pathway of its reaction with VRC (B).

3.5. Validation of the Proposed Assay

Assay validation was conducted according to The International Conference of Harmonization (ICH) guidelines for validation of analytical procedures [23].

3.5.1. Calibration and Sensitivity

Under the optimum reaction conditions (Table 1), the calibration curve for the determination of VRC by its reaction with CHDD was constructed by plotting the absorbances as a function of the corresponding concentrations. The regression equation for the results was A = 0.064 + 0.0118 C (r = 0.9986), where A is the absorbance at 540 nm, C is the concentration of VRC in µg/mL in the range of 5 - 100 µg/mL, and r is the correlation coefficient. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the following formula: LOD or LOQ= ×SDa/b, where ×= 3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The LOD and LOQ were found to be 2.29 and 6.95 µg/mL, respectively.

The parameters for the analytical performance of the proposed method are summarized in (Table 2).

Table 2. Quantitative parameters for the analytical performance of the proposed microwell-plate spectrophotometric assay for VRC.

Parameter	Value
Linear range (µg/mL)	5 – 100
Intercept (a)	0.0064
Standard deviation of intercept	0.0082
Slope	0.0118
Standard deviation of slope	0.0024
Correlation coefficient	0.9986
Limit of detection, LOD (µg/mL)	2.29
Limit of quantification, LOQ (µg/mL)	6.95

3.5.2. Precision

The intra-assay precision of the proposed method was determined on samples of drug solutions at varying concentration levels (Table 3) by analyzing 6 replicates of each concentration as a batch in a single assay run. The inter-assay precision was determined by analysis the same samples as duplicates in three consecutive days. The relative standard deviations (RSD) did not exceed 2 % (Table 3) proving the high precision of the proposed assay for the routine application in the analysis of VRC in quality control laboratories.

Table 3. Precision of the proposed microwell-plate spectrophotometric assay at different concentrations of VRC.

VRC Concentration (μg/mL)	Relative standard deviation	
	Intra-assay, n = 6	Inter-assays, n = 6
10	1.58	1.94
40	0.92	1.42
80	1.55	1.97

3.5.3. Accuracy and Interference Liabilities

The accuracy of the proposed method was evaluated by the recovery studies. The recovery values were 97.25 - 101.05 ± 1.24 - 1.94% (Table 4), indicating the accuracy of the proposed assay. Before proceeding with the analysis of VRC in its dosage forms, interference liabilities were carried out to explore the effect of inactive ingredients that might be added during VRC formulation [24]. Samples were prepared by mixing known amount (1 mg) of VRC with 10 mg of microcrystalline cellulose, 10 mg of calcium hydrogen phosphate anhydrous, 5 mg of croscarmellose sodium, 5 mg of silica-colloidal anhydrous and 5 mg magnesium stearate. These laboratory-prepared samples were analyzed by the proposed method applying the general recommended procedure. The average recovery value was of 99.27 ± 1.38% (Table 5). These data confirmed the absence of interference from any of the inactive ingredients with the determination of VRC by the proposed method.

Table 4. Recovery studies for determination of VRC by the proposed microwell-plate spectrophotometric assay.

Sample number	VRC		Recovery (% ± SD)[a]
	Nominal (μg/mL)	Found (μg/mL) [a]	
1	10	9.73	97.25 ± 1.54
2	40	40.42	101.05 ± 1.24
3	80	80.19	100.24 ± 1.94

[a] Values are mean of three determinations.

Table 5. Analysis of VRC in presence of the excipients that are present in its tablets by the proposed microwell-plate spectrophotometric assay.

Excipient	Recovery (% ± SD) [a]
Microcrystalline cellulose (10)[a]	98.51 ± 1.54
Crosscarmellose sodium (10)	101.05 ± 1.08
Calcium hydrogen phosphate anhydrous (5)	97.85 ± 1.48
Silica-colloidal anhydrous (5)	98.52 ± 1.04
Magnesium stearate (5)	100.41 ± 1.82
Average ± SD	99.27 ± 1.38

[a] Values are mean of three determinations.

[b] Figures in parenthesis are the amounts (in mg) that were added per 1 mg of VRC.

3.5.4. Robustness

Robustness was examined by evaluating the influence of small variation in the assay variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in one of the parameters did not significantly affect the procedures; recovery values were 96.82 - 102.04% ± 1.25 - 1.94%. This indicated the reliability of the proposed assay during its routine application for the analysis of VRC.

3.6. Application for Analysis of VRC in Tablets

It is evident from the above-mentioned results that the proposed method gave satisfactory results with VRC in bulk powder. Thus, its pharmaceutical dosage form (Champix® tablets) was subjected to the analysis of their VRC contents by the proposed method. The percentage found from the label claim was 98.80 ± 1.30%. This result was compared with those obtained by a reported method [7]; the label claim percentage was 97.30 ± 0.79%, with respect to the accuracy (by t-test), and precision (by F-test). It was found that the calculated t- and F-values (1.95 and 2.70 for t- and F-value, respectively) were lower than the tabulated ones (2.78 and 6.39 for t- and F-value, respectively). This indicated that there were no significant differences between the means and variance between the two methods in terms of the accuracy and precision.

4. Conclusions

This study described the successful development of a novel microwell-plate spectrophotometric assay for the accurate determination of VRC in its bulk and tablets based on its derivatization with CHDD as a chromogenic reagent. The assay described herein offered the following advantages:

Use of an inexpensive, stable reagent with excellent shelf life, and available in any pharmaceutical QC laboratory.

Use of minimum volumes of reagent and organic solvents, accordingly reduction in the analysis cost and exposures of the analysts to the toxic effects of organic solvents.

Providing a high throughput analytical methodology that can facilitate the processing of large number of samples in a relatively short time. This property was attributed to the use of multi-channel pipettes for efficient dispensing of the solutions, carrying out the analytical reaction in 96-well plates (as reaction vessels), and measuring the color signals in the 96 wells at ~ 30 seconds by the plate reader.

Although the proposed assay was developed and validated for VRC, however, it is also anticipated that the same methodology could be used for essentially any analyte that can exhibit the same substitution reaction with CHDD reagent.

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