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RESEARCH ARTICLE

Sensitive Spectrophotometric Assay of Two Phosphodiesterase Type 5-Inhibitors in Pure and Dosage forms Using Potassium Permanganate

RAGAA EL SHIEKH[1], ALAA S. AMIN[2], EMAN M. HAFEZ[1] and AYMAN A. GOUDA[1*]

[1]Chemistry Department, Faculty of Sciences, Zagazig University, Zagazig, 44519, Egypt
[2]Chemistry Department, Faculty of Sciences, Benha University, Benha, Egypt
aymangouda77@gmail.com

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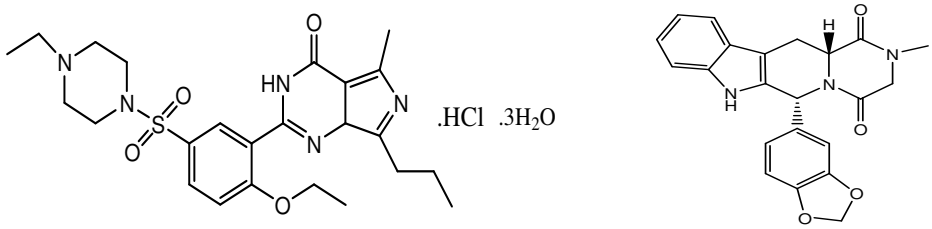
Abstract: Rapid, simple and sensitive and validated spectrophotometric methods have been developed for the assay of two phosphodiesterase type 5-inhibitors; vardenafil HCl (VARD) and tadalafil (TDF) in pure and dosage forms. The proposed methods were based on the oxidation of both drugs by a known excess of potassium permanganate ($KMnO_4$) in acidic medium and estimating the unreacted permanganate by the reaction with a fixed amount of three dyes, amaranth, indigo carmine and methylene blue, in the same acid medium followed by measuring the absorbance at λ_{max}=520, 610 and 664 nm, respectively. Different variables affecting the reaction were studied and optimized. The beer's law is obeyed in the concentration ranges of 2.0-12, 2.0-15 and 2.0-12 µg mL^{-1} for VARD and 2.0-15, 2.0-20 and 2.0-12 µg mL^{-1} for TDF using amaranth, indigo carmine and methylene blue methods, respectively with a correlation coefficient ≥ 0.9992. The apparent molar absorptivity values are in the range 2.0956×10^4, 1.2138×10^4 and 1.7502×10^4 L mol^{-1} cm^{-1} for VARD and 1.0769×10^4, 0.7922×10^4 and 1.0918×10^4L mol^{-1} cm^{-1} for TDF, using amaranth, indigo carmine and methylene blue methods, respectively. The limits of detection and quantification are reported. Intra-day and inter-day accuracy and precision of the methods have been evaluated. No interference was observed from the common tablet excipients. The methods were successfully applied to the assay of VARD and TDF in tablet preparations and the results were statistically compared with those of the reported methods by applying Student's *t*-test and F-test. The reliability of the methods was further ascertained by performing recovery studies using the standard addition method.

Keywords: Spectrophotometry, VardenafilHCl, Tadalafil, Potassium permanganate, Oxidation reactions, Tablets

Introduction

Vardenafil hydrochloride (VARD)is designated chemically aspiperazine, 1-[[3-(1,4-dihydro-5-methyl-4-oxo-7-propylimidazo[5,1-f] [1,2,4]triazin-2-yl)-4-ethoxy-phenyl] sulfonyl]-4-ethyl-, monohydrochloride and tadalafil (TDF) is designated chemically as(6*R*-trans)-6-(1,3-benzodioxol-5-yl)- 2,3,6,7,12,12a- hexahydro-2-methyl-pyrazino [1', 2':1,6] pyrido[3,4-*b*]

indole-1,4-dione (Figure 1). VARD and TDF are widely used as a selective phosphodiesterase type 5- inhibitor (PDE5) in the management of erectile dysfunction[1,2]. Extensive literature survey revealed that the determination of VARD and TDF in pure and dosage forms are not official in any of the pharmacopoeias and therefore, require much more investigation.



Vardenafilhydrochloride (VARD) Tadalafil (TDF)

Figure 1. The chemical structure of vardenafil hydrochloride (VARD) and tadalafil (TDF)

Few reports for the determination of VARD in pure, tablet dosage forms and biological fluids have been developed with the help of a variety of analytical tools including high performance liquid chromatography (HPLC)[3-12], gas chromatography[13,14], capillary electrophoresis[15,16], electrochemical methods[17,18] and atomic emission spectrometry[19-21]. Several analytical methods have been reported for the estimation of TDF in biological fluids or pharmaceutical dosage forms include HPLC[22-34], liquid chromatography-tandem mass spectrometry with electrospray ionization[35–37], micellar electro kinetic capillary chromatography[38] and atomic emission spectrometry[20,21].

All the above methods developed for the quantification of VARD and TAD employed complex analytical instruments for their estimation mainly in bulk drug powders, tablet dosage forms and biological fluids. However, most of these methods are complex, require expensive experimental setup and skilled personnel, suffer from time-consuming procedures and are inaccessible to many laboratories in developing and under developed nations. In contrast, visible spectrophotometry is considered as the most convenient analytical technique in most quality control and clinical laboratories, hospitals and pharmaceutical industries for the assay of different classes of drugs in pure, pharmaceutical formulations and biological samples, due to its simplicity and reasonable sensitivity with significant economic advantages.

To the best of our knowledge, there are some methods have been reported for the quantification of VARD and TDF in commercial dosage forms using a spectrophotometric technique[38-50] (Table 1). However, these previously reported methods suffer from one or the other disadvantage such as poor sensitivity, depending on critical experimental variables, few methods require a rigid pH control and tedious and time-consuming liquid–liquid extraction step; some other methods have a relatively narrow dynamic linear range, involve a heating step, and/or use of expensive reagent or large amounts of organic solvents. For these reasons, it was worth while to develop a new, simple, cost effective and selective spectrophotometric method for the determination of VARD and TDF their pharmaceutical dosage forms.

The three dyes, amaranth, indigo carmine and methylene blue are well known for their high absorptivity and have been utilized for estimation of excess oxidant. The present work aims to develop a simple, rapid, sensitive, accurate, precise and cost-effective spectrophotometric methods for the estimation of two phosphodiesterase type 5-inhibitors, VARD and TDF in pure and dosage forms based on the discoloring redox reaction with an excess of $KMnO_4$ and the determination of unreacted oxidant by the decrease in absorbance of the dyes; amaranth, indigo carmine and methylene blue.

Table 1. Comparison between the report spectrophotometric method for determination of VARD and TDF

Method	Wavelength, nm	Beer's law $\mu g\ mL^{-1}$	Molar Absorptivity, $L\ mol^{-1}cm^{-1}$	Detection Limit, $\mu g\ mL^{-1}$	Remarks	References
VARD						
3-Methyl-2-benzothiazolinone hydrazone hydrochloride/$FeCl_3$	625	4.0-40	NA	0.044	Less sensitive, less stable species measured	39
4-Aminoantipyrine/potassium periodate	530	4.0-60	NA	0.035		
BCG	418	2.0-14	$2.471x10^4$	0.56	Required close pH control and involved extraction steps organic solvent is used	40
BCP	410	2.0-20	$1.302x10^4$	0.49		
BTB	417	1.0-12	$4.594x10^4$	0.27		
BPB	417	2.0-14	$3.284x10^4$	0.53		
MO	429	1.0-20	$2.48x10^4$	0.26		
$KMnO_4$ /(a) Amaranth	520	2.0-15	$2.0956x10^4$	0.59	Highly sensitive and selective, no heating orextraction step, Inexpensive instrumental setup, use of ecofriendly chemicals and aqueous system	Present work
(b) Indigocarmine	610	2.0-20	$1.2138x10^4$	0.48		
(c) Methylene blue	664	2.0-12	$1.7502x10^4$	0.56		
TDF						
Ce(IV)/ methyl orange	507	18-60	$1.0464x10^4$	10.5	Less sensitive	45
N-bromosuccinamide/indigo carmine	610	10-55	$1.4922x10^4$	5.3		
Ce(IV)/ Indigo carmine	610	11-50	$0.8119x10^3$	3.5	Less sensitive	46
Ce(IV)/ methylene blue	600	10-55	$0.8367x10^3$	2.3		
Bromocresol purple (BCP)	410	2.0-16	$1.332x10^4$	0.092	Less sensitive, involves pH control, extraction step	47
Methyl orange (MO)	425	2.0-20	$1.033x10^4$	0.11		
Bromothymol blue (BTB)	420	10-50	NA	2.23	Less sensitive, involves pH control, extraction step	48
Bromocresol green (BCG)	415	10-50	NA	2.36		
Isatin	665	2.0-10	$7.70x10^3$	NA	Less sensitive,use conc. H_2SO_4	49
Xanthydrol	640	4.0-20	$2.59x10^4$	NA		
3-Methyl-2-benzothiazoline hydrazone (MBTH)	676	2.0-12	NA	0.0157	Heating required	50
$KMnO_4$ /(a) Amaranth	520	2.0-12	$1.0769x10^4$	0.52	Highly sensitive and selective, no heating orextraction step, Inexpensive instrumental setup, use of ecofriendly chemicals and aqueous system	Present work
(b) Indigocarmine	610	2.0-15	$0.7922x10^4$	0.58		
(c) Methylene blue	664	2.0-12	$1.0918x10^4$	0.50		

NA: not available

Experimental

All absorption spectra were made using Varian UV-Vis spectrophotometer (Cary 100 Conc., Australia) equipped with 10 mm quartz cell was used for absorbance measurements. This spectrophotometer has a wavelength accuracy of ±0.2 nm with a scanning speed of 200 nm/min and a band width of 2.0 nm in the wavelength range of 200-900 nm.

Materials and reagents

All chemicals, solvents and reagents used in this work were of analytical reagent or pharmaceutical grade and all solutions were prepared fresh daily. Bidistilled water was used throughout the investigation.

Reference standard of pure drugs

Pharmaceutical grade VARD and TDF working standard was kindly supplied by their respective manufactures in Egypt, without any conflicts of interests in our submitted paper.

Pharmaceutical formulations

The following tablets were purchased from local commercial markets. Levitra tablets are labeled to contain 10 mg VARD per tablet (Bayer Health Care Pharmaceuticals, Germany). Powerecta tablets are labeled to contain 20 mg VARD per tablet (Eva Pharma Company Giza, Egypt). Verdenodeb tablets are labeled to contain 20 mg VARD per tablet (Debeiky Pharmaceutical, Cairo, Egypt). Cialis® tablets, labeled to contain 20 mg TDF per tablet (Eli Lilly, Australia). Snafi®tablets, labeled to contain 20 mg TDF per tablet (Saudi Pharmaceutical Industries & Medical Appliances Corporation (SPIMACO), Al-Qassim, Saudi Arabia.

Standard solutions

A stock standard solution (100 µg mL^{-1}) of VARD and (200 µg mL^{-1}) TDF was prepared by dissolving 10 and 20 mg of pure VARD and TDF, respectively in bidistilled water and methanol, respectively further diluted to 100 mL with the same solvent in a 100 mL measuring flask. The standard solutions were found stable for at least one week without alteration when kept in an amber colored bottle and stored in a refrigerator when not in use.

Reagents

Potassium permanganate ($KMnO_4$) ($5.0x10^{-4}$ mol L^{-1})

A stock solution of $5.0x10^{-4}$mol L^{-1} $KMnO_4$ was freshly prepared by dissolving 0.079 g of $KMnO_4$ (Sigma-Aldrich) in 10 mL of warm bidistilled water then completed to the mark in a 100 mL calibrated flask and standardized using sodiumoxalate[51] and kept in a dark bottle and a refrigerator when not in use.

Sulfuric acid (H_2SO_4)(2.0 mol L^{-1})

A 2.0 mol L^{-1} of H_2SO_4 was prepared by adding 10.8 mL of concentrated acid (Merck, Darmstadt, Germany, 98%) to bidistilled water, cooled to room temperature, transfer to 100 mL with measuring flask, diluted to the mark and standardized as recorded[52].

Dyes (1000 µg mL^{-1})

A stock solutions (1000 µg mL^{-1}) amaranth, indigo carmine and methylene blue were first prepared by dissolving accurately weighed 112 mg of each dye (Sigma-aldrish, 90% dye content) in bidistilled water and diluting to volume in a 100 mL calibrated flask. The solution was then diluted 5.0-fold and 10-fold to get the working concentration of 200 and 100 µg mL^{-1} of (amaranthor indigocarmine) and methylene blue, respectively.

Recommended general procedures

For VARD

Different aliquots (0.2-1.2 mL), (0.2-1.5 mL) and (0.2-1.2 mL) of a standard 100 μg mL^{-1} VARD solution using amaranth, indigo carmine and methylene blue methods, respectively, were transferred into a series of 10 mL calibrated flasks followed by adding 1.0 mL 2.0 mol L^{-1} H_2SO_4 and 1.5 mL of $KMnO_4$ solution ($5.0x10^{-4}$mol L^{-1}) were added successively. The flasks were stoppered, content mixed and the flasks were kept aside for 5.0 min with occasional shaking. Finally, 1.5 mL of (200 μg mL^{-1}) amaranth, indigo carmineor methylene blue solution was added to each flask and mixed well and then the volume was diluted to the mark with water. The decrease in color intensity of dyes were measure dspectrophotometrically after 3.0 min against a blank solution containing the same constituent except drug treated similarly, at their corresponding λ_{max} 520, 610 and 664 nm for amaranth, indigo carmine and methylene blue methods, respectively. The concentration range was determined in each case by plotting the concentration of VARD against absorbance at the corresponding maximum wavelengths.

For TDF

Different aliquots (0.2-1.5 mL), (0.2-2.0 mL) and (0.2-1.2 mL) of a standard 100 μg mL^{-1} TDF solution using amaranth, indigocarmine and methylene blue methods, respectively, were transferred into a series of 10 mL calibrated flasks followed by adding 1.0 mL 2.0 mol L^{-1} H_2SO_4 and 1.0 and 1.5 mL of $KMnO_4$ solution (5.0×10^{-4}mol L^{-1}) using methylene blue and (amaranth or indigocarmine), respectively were added successively. The flasks were stoppered, content mixed and the flasks were kept aside for 5.0 min with occasional shaking. Finally, 1.0, 1.2 and 1.5 mL of (200 μg mL^{-1}) amaranth, indigo carmine and methylene blue solution, respectively were added to each flask and mixed well and then the volume was diluted to the mark with water. The decrease in color intensity of dyes were measured spectrophotometrically after 3.0 min against a blank solution containing the same constituent except drug treated similarly, at their corresponding λ_{max} 520, 610 and 664 nm for amaranth, indigo carmine and methylene blue methods, respectively. The concentration range was determined in each case by plotting the concentration of TDF against absorbance at the corresponding maximum wavelengths.

Procedure for pharmaceutical formulations (tablets)

The contents of twenty tabletsof each drug were weighed accurately and ground into a fine powder. An accurate weight of the powdered tablets equivalent to 20 mg VARD was dissolved in bidistilled water or 20 mg TDF was dissolved in methanol with shaking for 5.0 min and filtered using a Whatman No. 42 filter paper. The filtrate was diluted to the mark with bidistilled water for VARD or methanol for TDF in a 100 mL measuring flask to give and 200 μg mL^{-1} stock solution of VARD or TDF for analysis by spectrophotometric methods. A convenient aliquot was then subjected to analysis by the spectrophotometric procedures described above to determine the nominal content of the tablets using the corresponding regression equation of the appropriate calibration graph.

Results and Discussion

Absorption spectra

The spectrophotometric method for the determination of VARD and TDF involves two steps namely:

1. Oxidation of the studied drugs with a known excess of $KMnO_4$ in acidic medium at room temperature (25±2 °C).
2. Determination of the residual $KMnO_4$ by reacting it with a fixed amount of amaranth, indigocarmine and methylene blue dyes and measuring the absorbance of dyes at λ_{max} 520, 610 and 664 nm for amaranth, indigocarmine and methylene blue methods, respectively (Figure 2).

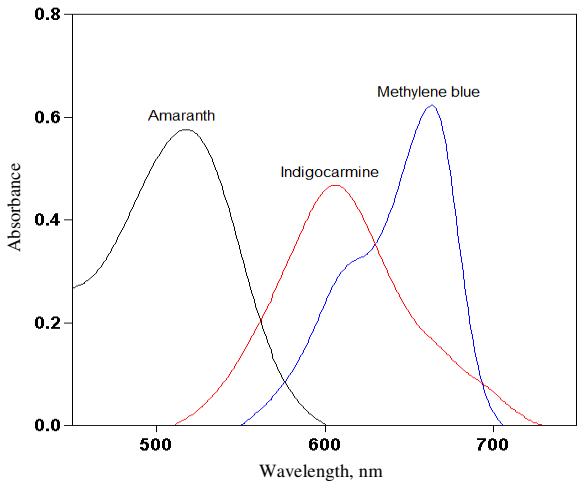
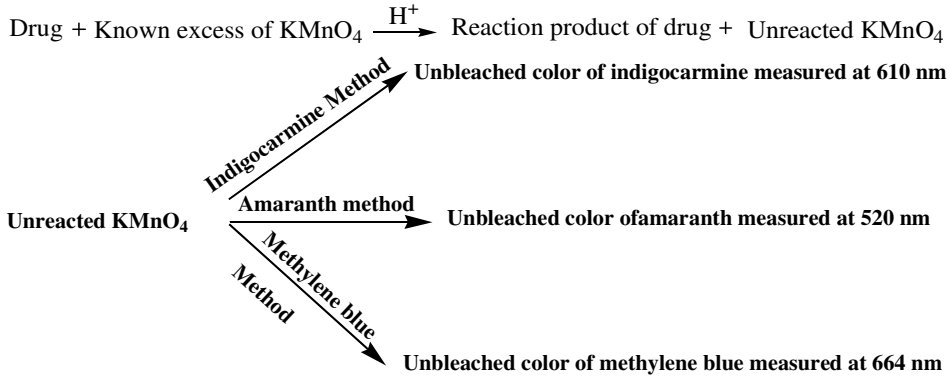


Figure 2. Absorption spectra for the unreacted oxidant that determined by reacting with a fixed amount of dyes and measuring the absorbance at 520, 610 and 664 nm for amaranth, indigocarmine and methylene blue methods, respectively in case of VARD

These methods make use of the bleaching action of $KMnO_4$ on the dyes, the decolorization being caused by the oxidative destruction of the dyes. VARD or TDF when added in increasing concentrations to a fixed concentration of $KMnO_4$ consumes the latter proportionally and there will be a concomitant decrease in the concentration of $KMnO_4$. When a fixed concentration of dye is added to decreasing concentrations of $KMnO_4$, a concomitant increase in the concentration of dye is obtained. Consequently, a proportional increase in the absorbance at the respective λ_{max} is observed with increasing concentrations of VARD or TDF. The tentative reaction scheme of spectrophotometric methods is shown in Scheme 1.



Scheme 1. Tentative reaction scheme for the proposed spectrophotometric methods

Optimization of variables

The optimum conditions for the assay procedures and color development for each method have been established by varying the parameters one at a time, keeping the others fixed and observing the effect produced on the absorbance of the colored species.

Effect of acid type and concentration

To study the effect of acid concentration, different types of acids were examined (H_2SO_4, H_3PO_4 and CH_3COOH) to achieve maximum yield of redox reaction. The results indicated that the sulphuric acid (H_2SO_4) was the most suitable acid with $KMnO_4$ as oxidant. Moreover, different volumes (0.2-3.0 mL) of 2.0 mol L^{-1} H_2SO_4 were tested and found to be 1.0 mL of 2.0 mol L^{-1} H_2SO_4 was ideal for the oxidation step in three methods and the same quantity of acid was employed for the estimation of the dye.

Effect of $KMnO_4$ concentration

The influence of the volume of $5.0x10^{-4}$ mol L^{-1} $KMnO_4$ on the reaction has been studied. It is apparent from Figure 3, that the absorbance increased with increasing volume of $5.0x10^{-4}$ mol L^{-1} $KMnO_4$ solution from (0.25-3.0 mL) and reached maximum when 1.5 mL of $KMnO_4$ was added to a total volume of 10 mL forVARD (Figure 3) and 1.0 and 1.5 mL of $KMnO_4$ solution were added to the total volume of 10 mL for TDF using methylene blue and (amaranth or indigocarmine), respectively.Therefore, it was found that maximum color intensity of the products was achieved with 1.5 mL of $5.0x10^{-4}$mol L^{-1} $KMnO_4$ for all measurements.

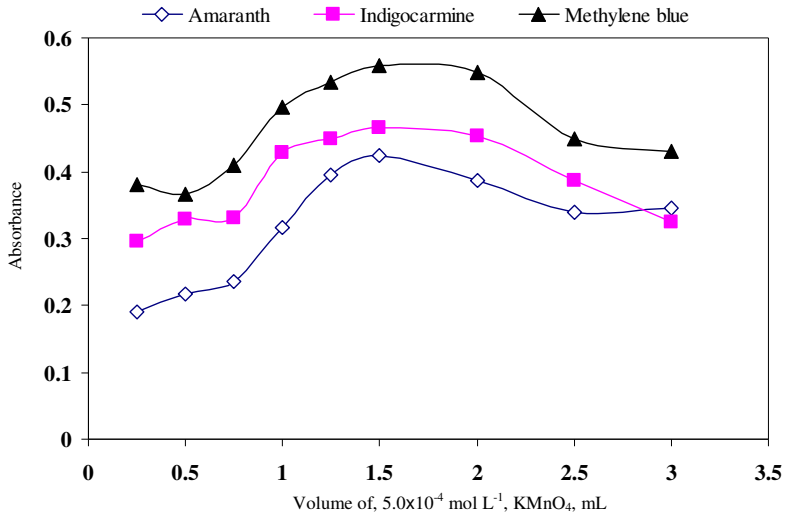


Figure 3. Effect of volume of $KMnO_4$ ($5.0x10^{-4}$ molL^{-1}) of the oxidation product of VARD with $KMnO_4$ and dyes in H_2SO_4 medium

Effect of dye concentration

The effect of amaranth, indigo carmine and methylene blue concentration on the intensity of the color developed was carried out to obtain the optimum concentration of dyes that produces the maximum and reproducible color intensity by reducing the residual of $KMnO_4$. The effect dye concentration was studied in the range of 0.25-3.0 mL of each dye (200 µg mL^{-1}). It was found that maximum color intensity of the oxidation products was achieved with 1.5

of each dye solution in case of VARD (Figure 4). Whereas, it was found that maximum color intensity of the oxidation products was achieved with 1.0, 1.2 and 1.5 mL of amaranth, indigo carmine and methylene blue dye solutions, respectively for TDF (Figure 5). The color was found to be stable up to 24 h.

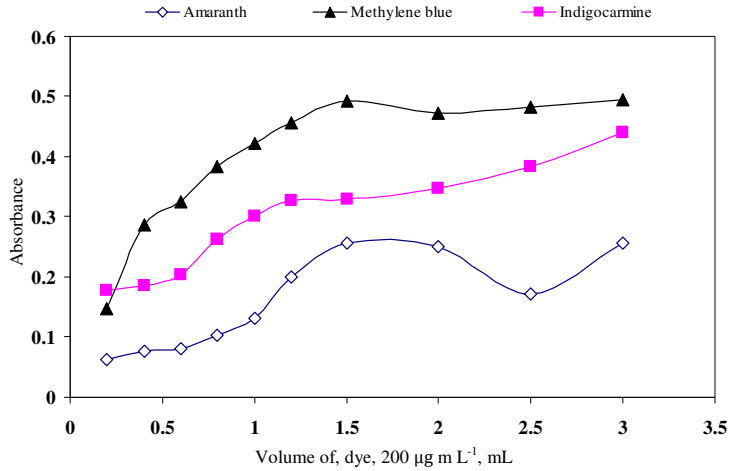


Figure 4. Effect of volume of dyes (200 µg mL^{-1}) of the oxidation product of VARD with $KMnO_4$ and dyes in H_2SO_4 medium

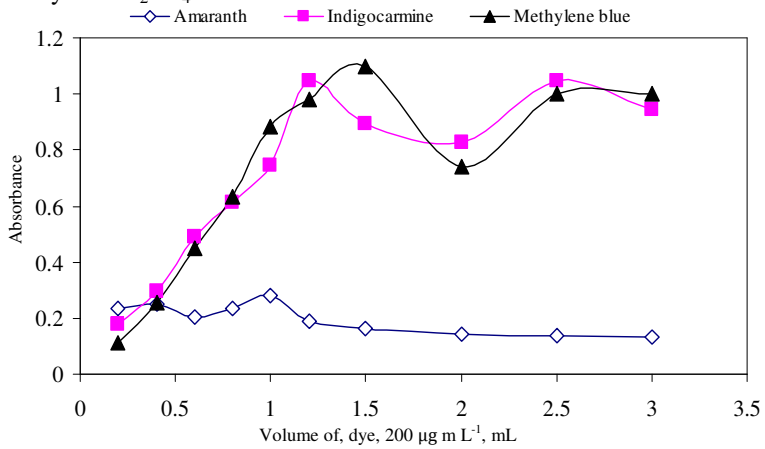


Figure 5. Effect of volume of dyes (200 µg mL^{-1}) of the oxidation product of TDF with $KMnO_4$ and dyes in H_2SO_4 medium

Effect of temperature and mixing time

The effect of temperature was studied by heating a series of sample and blank solutions at different temperatures ranging from 25 to 60 °C in water bath. It was found that raising the temperature does not accelerate the oxidation process and does not give reproducible results, so maximum color intensity was obtained at room temperature (25±2 °C). The effect of mixing time required completing oxidation of the studied drugs and for reducing the excess oxidant was studied by measuring the absorbance of sample solution against blank solution prepared similarly at various time intervals 2.0-20 min. It was found that the contact times gave constant and reproducible absorbance values at 5.0 min at room temperature (25±2 °C)

for both drugs. The time required for complete oxidation of the drug is not critical and any delay up to 15 min in the determination of unreacted $KMnO_4$ had no effect on the absorbance. After oxidation process, 3.0 min standing time was found necessary for the complete bleaching of the dye color by the residual $KMnO_4$ for both drugs and the absorbance of the unreacted dye was stable for at least 24 h, there after.

Effect of sequence of addition

The optimum sequence of addition was $KMnO_4$–H_2SO_4–drug–dye. Other sequences gave lower absorbance values under the same experimental conditions.

Stoichiometric ratio

The molar ratiomethod described by Yoe and Jones[53] was employed to determine the stoichiometry of drug, oxidant and dyes. The molar ratio between oxidant and dye [Dye]/[O] at the selected conditions was carried out, by keeping the concentration of the oxidant constant (1.5 mL of $5x10^{-4}$ mol L^{-1}) $KMnO_4$ and the drug (10 µg mL^{-1}) and variable volumes (0.1-2.0 mL) of dye ($5.0x10^{-4}$ mol L^{-1}) were added. The absorbance was measured at the suitable wavelength against blank solution prepared by the same manner. The absorbance values were then plotted against the molar ratio [Dye]/[O].

The molar ratio between the drug (VARD or TDF) and oxidant [D]/[O] at the selected conditions was carried out, by keeping the concentration of the oxidant constant (1.5 mL of $5x10^{-4}$ mol L^{-1}) $KMnO_4$ and (1.5 mL of $5.0x10^{-4}$ mol L^{-1}) dye and different volumes (0.1-2.0 mL) of the drug ($5.0x10^{-4}$ mol L^{-1}) were added. The absorbance was measured at the suitable wavelength against blank solution prepared by the same manner. The absorbance values were then plotted against the molar ratio [D]/[O]. Experimental results showed that the inflection of the lines at stoichiometric ratio (1:1) for [Dye]/[O]; (1.0:2.0)[D]/[O] and (1.0:2.0)[D]/[Dye] as shown in Table 2.

Method validation

The proposed methods have been validated for linearity, sensitivity, precision, accuracy, selectivity and recovery.

Linearity and sensitivity

Under the optimum conditions a linear correlation was found between absorbance at λ_{max} and the concentration of VARD and TDF in the ranges of 2.0-15 and 2.0-20 µg mL^{-1}, respectively. The calibration graph is described by the equation:

$$A = a + b\,C \quad (1)$$

Where A= absorbance, a= intercept, b= slope and C= concentration in µg mL^{-1}, obtained by the method of least squares. Correlation coefficient, intercept and slope of the calibration data are summarized in Table 2. For accurate determination, Ringbom concentration range[54] was calculated by plotting log concentration of drug in µg mL^{-1} against transmittance % from which the linear portion of the curve gives an accurate range of micro determination of VARD and TDF and represented in Table 2. Sensitivity parameters such as apparent molar absorptivity and Sandell's sensitivity values, as well as the limits of detection and quantification, were calculated as per the current ICH guidelines[55] and illustrated in Table 2. The high molar absorptivity and lower Sandell sensitivity values reflect the good and high sensitivity of the proposed methods. The validity of the proposed methods was evaluated by statistical analysis[56] between the results achieved from the

proposed methods and that of the reported method. Regarding the calculated Student's *t*-test and variance ratio *F*-test (Table 2), there is no significant difference between the proposed and reported methods[40,47] regarding accuracy and precision.

Table 2. Analytical and regression parameters of proposed oxidation spectrophotometric methods for determination of VARD and TDF

Parameters	VARD			TDF		
	Amaranth	Indigocarmine	Methylene blue	Amaranth	Indigocarmine	Methylene blue
Beer's law limits, µg mL^{-1}	2.0-12	2.0-15	2.0-12	2.0-15	2.0-20	2.0-12
Ringboom limits, µg mL^{-1}	4.0-10	4.0-12	4.0-10	4.0-12	4.0-16	4.0-10
Molar absorptivity, $x10^4$ L mol^{-1} cm^{-1}	2.0956	1.2138	1.7502	1.0769	0.7922	1.0918
Sandell sensitivity, ng cm^{-2}	26.80	46.35	32.08	36.16	49.15	35.67
Regression equation[a]						
Intercept (a)	0.0056	0.0022	0.0014	0.0043	0.0044	0.0008
Standard deviation of intercept (S_a)	0.009	0.023	0.016	0.009	0.02	0.008
Slope (b)	0.0358	0.0211	0.0307	0.0262	0.0194	0.0281
Standard deviation of slope (S_b)	0.018	0.015	0.027	0.013	0.017	0.012
Correlation coefficient, (r)	0.9993	0.9994	0.9999	0.9991	0.9992	0.9998
Mean ± SD	100.81±1.06	99.45±0.94	100.42±0.89	99.51±1.17	99.73±1.41	98.75±1.36
RSD%	1.05	0.95	0.89	1.18	1.41	1.38
RE%	1.10	0.99	0.93	1.23	1.48	1.45
Limit of detection, µg mL^{-1}	0.59	0.48	0.56	0.52	0.58	0.50
Limit of quantification, µg mL^{-1}	1.97	1.60	1.87	1.73	1.93	1.67
Calculated *t*-value[b]	1.03	1.66	0.34	0.19	0.08	1.08
Calculated *F*-value[b]	3.58	2.82	2.53	1.21	1.19	1.11
[Dye]/[O]	1:1	1:1	1:1	1:1	1:1	1:1
[D]/[Dye]	1:2	1:2	1:2	1:2	1:2	1:2
[D]/[Dye]	1:2	1:2	1:2	1:2	1:2	1:2

[a]$A = a + bC$, where C is the concentration in µg mL^{-1}, A is the absorbance units, a is the intercept, b is the slope. [b]The theoretical values of t and F are 2.57 and 5.05, respectively at confidence limit at 95% confidence level and five degrees of freedom (p= 0.05).

The limits of detection (LOD) and quantification (LOQ) were calculated according to the same guidelines using the formulas[55,56]:

$$LOD=3.3\sigma/s \text{ and } LOQ=10\sigma/s \quad (2)$$

Where σ is the standard deviation of five reagent blank determinations and s is the slope of the calibration curve.

Accuracy and precision

In order to evaluate the precision of the proposed methods, solutions containing three different concentrations of VARD and TDF were prepared and analyzed in six replicates. The analytical results obtained from this investigation are summarized in Table 3 & 4. Lower values of the relative standard deviation (R.S.D%) and percentage relative error (R.E%) indicate the precision and accuracy of the proposed methods. The percentage relative error is calculated using the following equation:

$$\%R.E = \left[\frac{found - taken}{taken}\right] x100 \quad (3)$$

The assay procedure was repeated six times and percentage relative standard deviation (R.S.D%) values were obtained within the same day to evaluate repeatability (intra-day precision) and over five different days to evaluate intermediate precision (inter-day precision).

For the same concentrations of drugs inter- and intra-day accuracy of the methods was also evaluated. The percentage recovery values with respect to found concentrations of each drug were evaluated to ascertain the accuracy of the methods. The recovery values close to 100% as compiled in Table 3 & 4 shows that the proposed methods are very accurate.

Table 3. Results of intra-day and inter-day accuracy and precision study for VARD obtained by the proposed methods

Method	Taken μg mL^{-1}	Recovery %	Precision RSD %[a]	Accuracy RE %	Confidence Limit[b]
			Intra-day		
Amaranth	4.0	99.00	0.56	-1.0	3.960±0.023
	8.0	99.30	0.74	-0.70	7.944±0.062
	12	99.20	1.02	-0.80	11.904±0.127
Indigocarmine	4.0	99.10	0.67	-0.90	3.964±0.028
	8.0	98.90	1.10	-1.10	7.912±0.091
	12	100.20	1.25	0.20	12.024±0.158
Methylene blue	4.0	99.40	0.80	-0.60	3.976±0.033
	8.0	99.00	0.97	-1.0	7.920±0.081
	12	99.70	1.19	-0.30	11.964±0.149
			Inter-day		
Amaranth	4.0	99.60	0.47	-0.40	3.984±0.02
	8.0	99.80	0.82	-0.20	7.984±0.069
	12	99.00	1.15	-1.0	11.88±0.143
Indigocarmine	4.0	99.50	0.63	-0.50	3.980±0.026
	8.0	99.40	0.96	-0.60	7.952±0.080
	12	100.50	1.30	0.50	12.06±0.165
Methylene blue	4.0	100.30	0.75	0.30	4.012±0.032
	8.0	99.40	1.10	-0.60	7.952±0.087
	12	99.10	1.60	-0.90	11.892±0.20

[a]RSD%, percentage relative standard deviation; RE%, percentage relative error. [b]Mean ± standard error

Robustness and ruggedness

For the evaluation of method robustness, volume of H_2SO_4 was slightly altered (1.0±0.2 mL) and the reaction timewas slightly varied deliberately (5.0±2.0 min) (after adding $KMnO_4$) in the three methods for each drug. The analysis was performed with altered conditions by taking three different concentrations of drugs and the methods were found to remain unaffected as shown by the RSD values in the ranges of 0.75-2.40% and 0.70-2.20% for VARD and TDF, respectively. Methods ruggedness was expressed as the RSD of the same procedure applied by three different analysts as well as using three different instruments (spectrophotometers). The inter-analysts RSD were in the ranges 0.80-2.20% and 0.60-1.95% for VARD and TDF, respectively, whereas the inter-instruments RSD ranged from 0.80-2.40% and 0.90-2.30% for VARD and TDF, respectively suggesting that the developed methods were rugged. The results are shown in Table 5.

Table 4. Results of intra-day and inter-day accuracy and precision study for TDF obtained by the proposed methods

Method	Taken μg mL^{-1}	Recovery %	Precision RSD % [a]	Accuracy RE %	Confidence Limit [b]
			Intra-day		
Amaranth	4.0	99.30	0.42	-0.70	3.972±0.018
	8.0	99.60	0.76	-0.40	7.968±0.064
	12	99.40	0.90	-0.60	11.928±0.113
Indigocarmine	5.0	99.10	0.68	-0.90	4.955±0.035
	10	99.00	1.10	-1.0	9.90±0.114
	15	100.40	1.35	0.40	15.06±0.213
Methylene blue	4.0	100.30	0.70	0.30	4.012±0.029
	8.0	99.80	0.90	-0.20	7.984±0.075
	12	99.20	1.40	-0.80	11.904±0.175
			Inter-day		
Amaranth	4.0	99.30	0.53	-0.70	3.972±0.022
	8.0	99.50	0.69	-0.50	7.960±0.058
	12	99.10	1.08	-0.90	11.892±0.135
Indigocarmine	5.0	100.20	0.49	0.20	5.01±0.026
	10	99.30	0.78	-0.70	9.93±0.081
	15	100.30	1.25	0.30	15.045±0.197
Methylene blue	4.0	99.20	0.60	-0.80	3.968±0.025
	8.0	99.60	0.88	-0.40	7.968±0.074
	12	99.00	1.32	-1.0	11.88±0.165

[a]RSD%, percentage relative standard deviation; RE%, percentage relative error. [b]Mean ± standard error

Recovery studies

To ascertain the accuracy, reliability and validity of the proposed methods, recovery experiment was performed through standard addition technique. This study was performed by spiking three different levels of pure drugs (50, 100 and 150% of the level present in the tablet) to a fixed amount of drugs in tablet powder (pre-analysed) andthe total concentration was found by the proposed methods. The determination with each level was repeated three times and the percent recovery of the added standard was calculated from:

$$\% \text{ Recovery} \frac{[C_F - C_T]}{C_p} x100 \quad (4)$$

Where $C_F$is the total concentration of the analyte found, C_T is a concentration of the analyte present in the tablet preparation; C_P is a concentration of analyte (pure drugs) added to tablets preparations. The results of this study presented in Table 6 revealed that the accuracy of the proposed methods was unaffected by the various excipients present in tablets which did not interfere in the assay.

Table 5. Results of method robustness and ruggedness (all values in RSD%) studies for VARD and TDF

Methods	Nominal amount concentration, µg mL^{-1}	RSD%			
		Robustness		Ruggedness	
		Variable alerted[a]			
		Acid volume (n=3)	Reaction time (n=3)	Different analysts (n=3)	Different instruments (n=3)
		VARD			
Amaranth	4.0	1.20	0.75	0.80	0.90
	8.0	1.62	1.25	1.50	1.30
	12	2.10	1.80	1.90	2.30
Indigocarmine	4.0	1.10	0.90	1.20	0.80
	8.0	1.40	1.70	1.54	1.30
	12	2.20	2.10	1.90	2.30
Methylene blue	4.0	1.15	0.95	0.80	1.05
	8.0	1.80	1.50	1.60	1.70
	12	2.40	2.00	2.20	2.40
		TDF			
Amaranth	4.0	0.80	0.70	0.90	1.10
	8.0	1.25	1.40	1.30	1.60
	12	1.90	2.15	1.80	2.20
Indigocarmine	5.0	0.75	0.95	0.60	1.20
	10	1.60	1.20	1.10	1.70
	15	2.10	1.70	1.75	2.30
Methylene blue	4.0	0.92	0.84	1.05	0.90
	8.0	1.45	1.30	1.55	1.30
	12	2.20	2.00	1.95	2.15

[a]*Volume of (5.0 mol L^{-1}) HCl is (1.0±0.2 mL) and reaction time is (5.0±2.0 min) (after adding NBS) were used*

Application of pharmaceutical formulations (tablets)

The proposed methods were applied to the determination of VARD and TDF in pharmaceutical formulations (tablets). The results in Table 7 showed that the methods are successful for the determination of VARD and TDF and that the excipients in the dosage forms do not interfere. A statistical comparison of theresults obtained from the assay of VARD and TDF by the proposed methods and the reported methods[40,47] for the same batch of material is presented in Table 7. The results agree well with the label claim and also were in agreement with the results obtained by the reported methods[40,47]. When the results were statistically compared with those of the reported methods by applying the Student's *t*-test for

accuracy and *F*-test for precision, the calculated *t*-value and *F*-value at 95% confidence level did not exceed the tabulated values for five degrees of freedom[56]. Hence, no significant difference between the proposed methods and the reported methods at the 95% confidence level with respect to accuracy and precision.

Table 6. Results of recovery experiments by standard addition method for the determination of VARD and TDF in tablets using the proposed methods

Samples	Taken drug in tablet μg mL^{-1}	Pure drug Added μg mL^{-1}	Amaranth		Methylene blue		Indigocarmine	
			Total found μg mL^{-1}	Recovery[a] (%) ± SD	Total found μg mL^{-1}	Recovery[a] (%) ± SD	Total found μg mL^{-1}	Recovery[a] (%) ± SD
Levitra	4.0	2.0	5.976	99.60±0.40	5.952	99.20±0.65	5.988	99.80±0.40
tablets	4.0	4.0	7.976	99.70±0.72	7.96	99.50±0.87	7.928	99.10±0.57
(10 mg)	4.0	6.0	10.02	100.20±0.86	9.90	99.00±1.08	10.05	100.50±0.73
Powerecta	4.0	2.0	6.012	100.20±0.39	5.964	99.40±0.54	5.958	99.30±0.55
tablets	4.0	4.0	8.064	100.80±0.58	7.976	99.70±0.67	8.072	100.90±0.70
(20 mg)	4.0	6.0	9.89	98.90±0.63	10.0	100.00±0.86	9.91	99.10±0.90
Verdenodeb	4.0	2.0	5.94	99.00±0.60	6.048	100.80±0.50	5.94	99.00±0.85
tablets	4.0	4.0	8.056	100.70±0.88	7.968	99.60±0.76	7.936	99.20±0.96
(20 mg)	4.0	6.0	9.96	99.60±1.10	9.91	99.10±1.25	10.05	100.50±1.30
	Taken drug in tablet μg mL^{-1}	Pure drug Added μg mL^{-1}	Amaranth		Methylene blue		Orange G	
			Total found μg mL^{-1}	Recovery[a] (%) ± SD	Total found μg mL^{-1}	Recovery[a] (%) ± SD	Total found μg mL^{-1}	Recovery[a] (%) ± SD
Cialis®	4.0	2.0	5.964	99.40±0.65	6.03	100.50±0.35	5.952	99.20±0.65
tablets	4.0	4.0	8.016	100.20±0.90	7.968	99.60±0.60	8.056	100.70±0.90
(20 mg)	4.0	6.0	9.98	99.80±1.17	10.03	100.30±1.10	10.10	101.00±1.40
Snafi®	4.0	2.0	6.036	100.60±0.52	5.97	99.50±0.44	5.982	99.70±0.63
tablets	4.0	4.0	7.928	99.10±0.85	7.888	98.60±1.10	8.04	100.50±0.80
(20 mg)	4.0	6.0	10.13	101.30±1.30	9.95	99.50±1.50	9.93	99.30±1.20

[a]*Average of six determinations*

Table 7. Results of analysis of tablets by the proposed methods for the determination of VARD and TDF and statistical comparison with the reference methods

Samples	Recovery[a], % ± SD			
	Proposed Methods			Reported methods
	Amaranth	Methylene blue	Indigo carmine	
Levitra tablets (10 mg VARD)	99.30±0.35	99.60±0.45	100.40±0.80	99.92±0.64[40]
t-value[b]	1.9	0.91	1.04	
F-value[b]	3.34	2.02	1.56	
Powerectatablets (20 mg VARD)	100.50±0.80	99.50±0.30	99.20±0.90	99.90±0.67[40]
t-value[b]	1.28	1.21	1.39	
F-value[b]	1.42	4.98	1.80	
Verdenodebtablets (20 mg VARD)	99.10±0.85	99.70±0.50	99.80±0.93	99.50±0.72[40]
t-value[b]	0.8	0.51	0.57	
F-value[b]	1.39	2.07	1.66	

	Amaranth	Methylene blue	Orange G	
Cialis® tablets (20 mg TDF)	100.40±0.30	99.40±0.78	100.10±0.74	99.79±0.56[47]
t-value [b]	2.14	0.90	0.74	
F-value [b]	3.48	1.94	1.74	
Snafi®tablets (20 mg TDF)	99.30±0.68	100.20±0.75	99.43±0.40	99.60±0.51[47]
t-value [b]	0.78	1.47	0.58	
F-value [b]	1.77	2.16	1.62	

[a]*Average of six determinations.* [b]*The theoretical values of t and F are 2.571 and 5.05, respectively at confidence limit at 95% confidence level and five degrees of freedom (p = 0.05)*

Conclusion

Three new, useful simple, rapid, and cost-effective spectrophotometric methods have been developed for determination of VARD and TDF in bulk drugs and in their tablets using $KMnO_4$ as oxidizing agent and validated as per the current ICH guidelines. The present spectrophotometric methods are characterized by simplicity of operation, high selectivity, comparable sensitivity, low-cost instrument, they do not involve any critical experimental variable and are free from tedious and time-consuming extraction steps and use of organic solvents unlike many of the previous methods reported for VARD and TDF. The assay methods have some additional advantages involve less stringent control of experimental parameters such as the stability of the colored system, accuracy, reproducibility, time of analysis, temperature independence and cheaper chemicals. These advantages encourage the application of the proposed methods in routine quality control analysis of VARD and TDF in pure and dosage forms.

Conflict of interest

The authors declare that they have no conflict of interests with the company name used in the paper.

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