Simple Spectrophotometric Methods for the Determination of Two Phosphodiesterase Type 5-Inhibitors in Pure and Tablets Dosage Forms Using *N*-Bromosuccinimide as an Oxidant

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Abstract: Three simple, sensitive spectrophotometric methods were proposed for the quantification of two phosphodiesterase type 5-inhibitors**;** vardenafil HCl (VARD) and tadalafil (TDF) in pure forms as well as in tablets dosage forms. The methods use *N*-bromosuccinimide (NBS) as an analytical reagent and three dyes, amaranth methylene blue, and indigocarmine or orange G, as auxiliary reagents. The three methods are based on oxidation reaction of VARD or TDF with a known excess of *N*-bromosuccinimide (NBS) in acid medium, followed by determination of unreacted NBS by the reaction with a fixed amount of three dyes, amaranth, methylene blue and indigocarmineor orange G followed by the measurement of the absorbance at 520, 664 and 610 or 478 nm, respectively. Under the optimum conditions, the three methods are applicable over the concentration ranges of 1.0-16, 1.0-12 and 1.0-10 μgmL^{-1} for VARD using amaranth methylene blue and indigocarmine methods, respectively and 2.0-12, 2.0-15 and 1.0-10 μgmL^{-1} for TDF using amaranth methylene blue and orange G, respectively. The molar absorptivities, Sandell's sensitivity values, correlation coefficients, 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 tablets preparations and the results were statistically compared with those of the reference 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, *N*-bromosuccinimide, Tablets

Introduction

Vardenafilhydrochloride (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-, monohydrochlorideand 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.
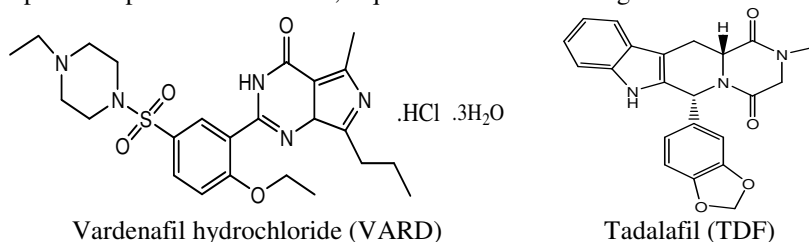

Vardenafil hydrochloride (VARD)   Tadalafil (TDF)

Figure 1. The chemical structure of vardenafil HCl (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 electrokinetic capillary chromatography[38] and atomic emission spectrometry[20,21].

All the above methods developed for the quantification of VARD and TDF 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 in 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 worthwhile to develop a new simple, cost effective and selective spectrophotometric method for the determination of VARD and TDF their pharmaceutical dosage forms.

From the foregoing paragraphs, it is clear that *N*-bromosuccinimide (NBS) despite its strong oxidizing power, versatility and high oxidation potential and stability in solution has not been applied for the assay of VARD and TDF in pure forms and tablets.

The present investigation aims to develop for the first time sensitive and cost-effective methods for the determination of VARD and TDF in pure and dosage forms using spectrophotometric techniques. The methods employ *N*-bromosuccinimide which acts as brominating agent and four dyes; amaranth, methylene blue indigocarmine or orange G, as auxiliary chromogenic reagents.

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

Method	Wavelength, nm	Beer's Law µg mL^{-1}	Molar Absorptivity, L mol^{-1}cm^{-1}	Detection Limit µg mL^{-1}	Remarks	[Reference]s
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		
Bromocresol green (BCG)	418	2.0-14	2.471x10^4	0.56		
Bromocresol purple (BCP)	410	2.0-20	1.302x10^4	0.49	Required close pH control and involved extraction steps organic solvent is used	[40]
Bromothymol blue (BTB)	417	1.0-12	4.594x10^4	0.27		
Bromophenol blue (BPB)	417	2.0-14	3.284x10^4	0.53		
Methyl orange (MO)	429	1.0-20	2.48x10^4	0.26		
NBS /(a) Amaranth	520	1.0-16	0.9717x10^4	0.29	Highly sensitive and selective, no heating orextraction step, Inexpensive instrumental setup, use of ecofriendly chemicals, and aqueous system	Present work
(b) Methylene blue	664	1.0-12	2.5114x10^4	0.27		
(c) Indigocarmine	610	1.0-10	2.208x10^4	0.26		
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$_2$SO$_4$	[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]
NBS /(a) Amaranth	520	2.0-12	0.9595x10^4	0.58	Highly sensitive and selective, no heating orextraction step, Inexpensive instrumental setup, use of ecofriendly chemicals and aqueous system	Present work
(b) Methylene blue	664	2.0-15	1.8077x10^4	0.55		
(c) Orange G	478	1.0-10	0.797x10^4	0.27		

NA: not available

The proposed methods have been demonstrated to be superior to the reported methods with respect to speed, simplicity, sensitivity, being accurate and precise, cost effectiveness, eco-friendliness and can be adopted by the pharmaceutical laboratories for industrial quality control.

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 bandwidth 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 were labeled to contain 10 mg VARD per tablet (Bayer HealthCare Pharmaceuticals, Germany). Powerecta tablets were labeled to contain 20 mg VARD per tablet (Eva PharmaCompany Giza, Egypt). Verdenodeb tablets were 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 µgmL^{-1}) of VARD and (200 µgmL^{-1}) TDF was prepared by dissolving 10 and 20 mg of pure VARD and TDF, respectivelyin 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

N-bromosuccinimide (NBS) (0.01 mol L^{-1})

A stocksolution of 0.01 mol L^{-1} NBS (Sigma-Aldrish) was freshly prepared by dissolving about 0.178 g of NBS in least amount of warm bidistilled water in a 100 mL measuring flask and then diluted to the mark with bidistilled water and standardized[51]. The solution was kept in an amber colored bottle and was diluted appropriately to get 100 µg mL^{-1} NBS for use in all methods. The NBS solution was stored in a refrigerator when not in use.

Potassium bromide (1.0% w/v)

A 1.0% w/v KBr solution was also prepared by dissolving 1.0 g of KBr in 100 mL water.

Hydrochloric acid (5.0 mol L^{-1})

A 5.0 mol L^{-1} of HCl was prepared by diluting 43 mL of concentrated acid (Merck, Darmstadt, Germany, Sp. gr. 1.18, 37%) to 100 mL with bidistilled water and standardized as recommended previously[52] prior to use.

Dyes (1000 µg mL^{-1})

A stock solutions of (1000 µg mL^{-1}) amaranth, methylene blue, indigocarmine and orange G 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 (amaranth, indigocarmine or orange G) and methylene blue, respectively.

Recommended general procedures

VARD

Different aliquots (0.1-1.6 mL), (0.1-1.2 mL), (0.1-1.2) and (0.1-1.0 mL) of a standard 100 µg mL^{-1} VARD solution using amaranth, methylene blue and indigocarmine methods, respectively, were transferred into a series of 10 mL calibrated flasks by means of a micro burette. To each flask 1.0 mL each of 5.0 mol L^{-1}HCl; 1.5 mL of NBS solution (100 µg mL^{-1}) and 1.0 mL of 1.0% (w/v) KBr 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 and 1.2 mL of (200 µgmL^{-1}) (amaranth or methylene blue) and indigocarmine solution, respectively were added to each flask and mixed well and then the volume was diluted to the mark with water. The absorbance of each solution was measured at 520, 664 and 610 nm for amaranth, methylene blue and indigocarmine methods, respectively, after 3.0 min against a reagent blank.

TDF

Different aliquots (0.2-1.2 mL), (0.2-1.5 mL) and (0.1-1.0 mL) of a standard 100 µg mL^{-1} TDF solution for amaranth, methylene blue and orange G methods, respectively, were transferred into a series of 10 mL calibrated flasks by means of a micropipette. To each flask 1.0 mL each of 5.0 mol L^{-1} HCl; 2.0 mL of NBS solution (100 µgmL^{-1}) and 1.0 mL of 1.0% (w/v) KBr were added successively. The flasks were stoppered, content mixed and the flasks were kept aside for 5.0 min with occasional shaking. Finally, 1.2 and 1.5 mL of (200 µgmL^{-1}) amaranth or methylene blue and orange G dyes solution, respectively was added to each flask and mixed well and then the volume was diluted to the mark with bidistilled water. The absorbance of each solution was measured at 520, 664 and 478 nm for amaranth, methylene blue and orange G methods, respectively, after 3.0 min against a reagent blank. In all methods, a standard graph was prepared by plotting the absorbance *versus* the concentration of drug. The concentration of the unknown was read from the calibration graph or computed from the regression equation derived using Beer's law data.

Procedure for pharmaceutical formulations (tablets)

The contents of twenty tablets of 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 µgmL^{-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. Determine the nominal content of the tablets using the corresponding regression equation of the appropriate calibration graph.

Results and Discussion

Absorption spectra

Many dyes are irreversibly destroyed to colorless species by oxidizing agents in acid medium[53]. The proposed spectrophotometric methods are based on the reaction between VARD or TDF and measured excess of NBS and subsequent determination of the latter by reacting it with a fixed amount of amaranth, methylene blue and indigocarmineor orange G dye and measuring the absorbance at 520, 664 and 610 or 478 nm (Figure 2). These methods make use of the bleaching action of NBS on the dyes, the decolorization being caused by the oxidative destruction ofthe dyes. VARD or TDF when added in increasing concentrations to a fixed concentration of NBS consumes the latter and there will be a concomitant decrease in the concentration of NBS. When a fixed concentration of either dye is added to decreasing concentrations of NBS, a concomitant increase in the concentration of dye is obtained. Consequently, a proportional increase in the absorbance at the respective $\lambda_{max}$is observed with increasing concentrations of VARD or TDF.
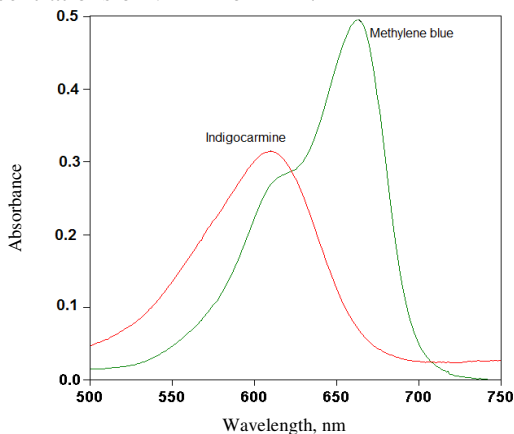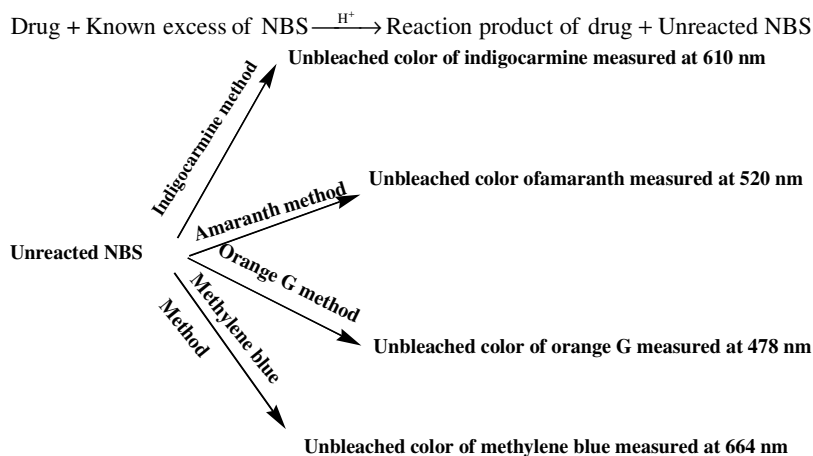

Figure 2. Absorption spectra for the unreacted oxidant that determined by reacting with a fixed amount of dyes and measuring the absorbance at 610 and 664 nm for indigocarmine and methylene blue methods, respectively in case of VARD

Chemistry of the reactions

NBS is a strong oxidizing or brominating agent and perhaps the most important positive bromine containing organic compound used for the determination of many pharmaceutical compounds[54–58]. It is also used for the specific purpose of brominating alkenes at the allylic position[59]. The analytical reactions involved two steps; the first one was concerned with the bromination of the investigated drugs with a known excess amount of NBS in hydrochloric acid medium. The second step involved the determination of the excess residual NBS via its reaction with a fixed amount of both amaranth, methylene blue, indigocarmine or orange G dyes and measuring the absorbance at the respective λ_{max}. The tentative reaction scheme of spectrophotometric methods is shown in Scheme 1. In all methods, the absorbance increased linearly with increasing concentration of drugs. The latter methods make use of the bleaching action of NBS on dyes, the discoloration being caused by the oxidative destruction of the dye.

Drug + Known excess of NBS $\xrightarrow{\text{H}^+}$ Reaction product of drug + Unreacted NBS

Unbleached color of indigocarmine measured at 610 nm

Unbleached color ofamaranth measured at 520 nm

Unreacted NBS

Indigocarmine method

Amaranth method

Orange G method

Methylene blue

Method

Unbleached color of orange G measured at 478 nm

Unbleached color of methylene blue measured at 664 nm

Scheme 1. Tentative reaction scheme for the proposed spectrophotometric methods

Selection of acid type and concentration

The reaction between VARD and TDF and NBS was performed in different acid media HCl, H_2SO_4, HNO_3 and CH_3-COOH solutions. Better results were suitable in hydrochloric acid medium. The effect of HCl concentration on the reaction between VARD and TDF and NBS was studied by varying the concentration of HCl keeping the concentrations of NBS and drug fixed. The reaction was found to be rapid yielding a constant absorbance with maximum sensitivity and stability when the HCl concentration was 5.0 mol L^{-1} and maintained in the range of 0.25-3.0 mL of HCl (5.0 mol L^{-1}) in a total volume of 10 mL.The results indicated that, at 1.0-3.0 mL of HCl (5.0 mol L^{-1}), there were almost same absorbance values were obtained in the presence of VARD and TDF, the absorbance values obtained were constant and were almost the same as those of the reagent blank. At the acid volumes less than 1.0 mL, reaction led to go slower and incomplete. Therefore, 1.0 mL of HCl (5.0 mol L^{-1}) was used though out the study for both drug.

Effect of NBS concentration

To investigate the optimum concentration of NBS, different concentrations of NBS were treated in the range of 0.25-3.0 mL with a fixed concentration dyes in HCl medium and the absorbance was measured at optimum wavelength. It was found that maximum color intensity of the products was achieved with 1.5and 2.0 mL of NBS (100 µgmL^{-1}) forVARD and TDF, respectively (Figure 3).

Effect of KBr concentration

The effect of KBr concentration was studied in the range of 0.5-2.5 mL. 1.0 mL of 1.0% (w/v) KBr was chosen as an optimum volume to accelerate the oxidation process.

Effect of dye concentration

The effect of amaranth, methylene blue, indigocarmine or orange G 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 NBS. 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 and 1.2 mL of (amaranth or methylene blue) and indigocarmine solution, respectively in case of VARD (Figure 4), but with 1.2 and 1.5 mL of (amaranth or methylene blue) and orange G dyes solution, respectively for TDF.
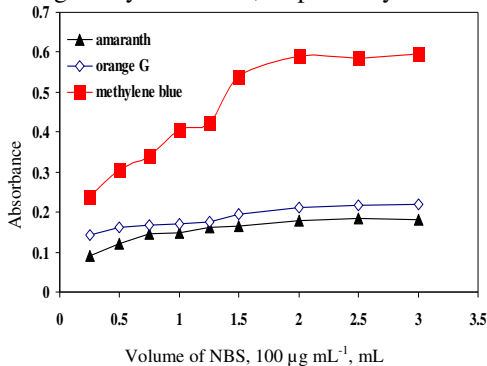

Figure 3. Effect of volume of NBS (100 μgmL^{-1}) of the oxidation product of TDF with NBS and dyes in HCl medium

Figure 4. Effect of volume of dyes (200 μgmL^{-1}) of the oxidation product of VARD with NBS and dyes in HCl 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 each drug. The time required for complete oxidation of the drug is not critical and any delay up to 15 min in the determination of unreacted NBS had no effect on the absorbance. A 3.0 min standing time was found necessary for the complete bleaching of the dye color by the residual NBS for each drug was found necessary for complete reduction of residual NBS by all dyesand the absorbance of the unreacted dye was stable for at least 6.0 h, thereafter

Effect of sequence of addition

The optimum sequence of addition was drug–HCl–NBS–KBr and then dye.Other sequences gave lower absorbance values under the same experimental conditions.

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 λ$_{max}$ and the concentration of VARD and TDF in the ranges of 1.0-16 μgmL^{-1} and 1.0-15, respectively. The calibration graph is described by the equation:

$$A = a + b\,C \tag{1}$$

Where A= absorbance, a= intercept, b= slope and C= concentration in μgmL^{-1}, obtained by the method of least squares. Correlation coefficient, intercept and slope of the calibration
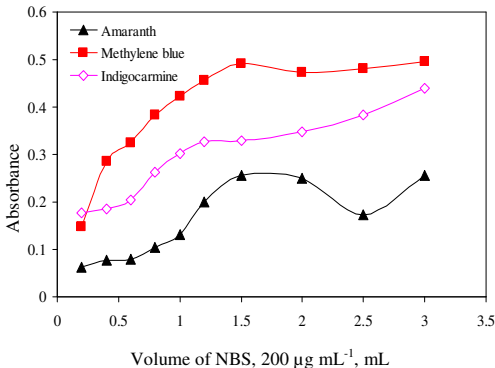
data are summarized in Table 2. For accurate determination, Ringbom concentration range[60] was calculated by plotting log concentration of drug in μgmL^{-1} against transmittance % from which the linear portion of the curve gives an accurate range of microdetermination of VARD and TDF and represented 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[61] 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[62] 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 method[40,47] regarding accuracy and precision. The limits of detection (LOD) and quantification (LOQ) were calculated according to the same guidelines using the formulas[61,62]:

$$LOD=3.3\sigma/s \text{ and } LOQ=10\sigma/s \tag{2}$$

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. 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:

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

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 shows that the proposed methods are very accurate.

Robustness and ruggedness

For the evaluation of method robustness, volume of HClwas slightly altered (1.0±0.2 mL) and the reaction time (after adding NBS, time varied was 5.0±2.0 min) were slightly varied deliberately 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 0.9-2.30% and 0.78-2.5% 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 0.84-2.50% and 0.85-2.10% for VARD and TDF, respectively, whereas the inter-instruments RSD ranged from 0.75-2.45% and 0.90-2.40% for VARD and TDF, respectively suggesting that the developed methods were rugged. The results are shown in Table 4.

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

Parameters	VARD			TDF		
	Amaranth	Methylene blue	Indigocarmine	Amaranth	Methylene blue	Orange G
Beer's law limits, µg mL^{-1}	1.0-16	1.0-12	1.0-10	2.0-12	2.0-15	1.0-10
Ringboom limits, µg mL^{-1}	3.0-13	3.0-10	2.0-8.0	4.0-10	4.0-12	2.0-8.0
Molar absorptivity, x 10^4 L mol^{-1} cm^{-1}	0.9717	2.5114	2.208	0.9595	1.8077	0.7970
Sandell sensitivity, ng cm^{-2}	57.79	22.36	25.43	40.58	21.54	48.86
Regression equation[a]						
Intercept (a)	0.0041	0.0059	0.0027	- 0.0009	0.004	0.0035
Standard deviation of intercept (S_a)	0.007	0.006	0.005	0.006	0.005	0.0048
Slope (b)	0.0156	0.042	0.038	0.0206	0.0454	0.0229
Standard deviation of slope (S_b)	0.011	0.008	0.009	0.008	0.007	0.0093
Correlation coefficient, (r)	0.9993	0.9997	0.9996	0.9997	0.9996	0.999
Mean ± SD	100.21±1.22	99.90±0.76	100.10±1.14	100.01±1.33	100.36±1.40	100.34±1.34
RSD%	1.22	0.76	1.14	1.33	1.40	1.34
RE%	1.28	0.80	1.20	1.40	1.47	1.41
Limit of detection, µg mL^{-1}	0.29	0.27	0.26	0.58	0.55	0.27
Limit of quantification, µg mL^{-1}	0.97	0.90	0.87	1.93	1.83	0.90
Calculated t-value [b]	0.08	0.85	0.28	0.52	0.90	0.91
Calculated F-value [b]	4.75	1.84	4.14	1.06	1.18	1.08

[a]$A = a + bC$, where C is the concentration in µgmL^{-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).

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	99.20	0.83	-0.80	3.968 ± 0.035
	8.0	100.30	1.14	0.30	8.024 ± 0.096
	12.0	99.60	1.55	-0.40	11.952 ± 0.194
Methylene blue	3.0	99.00	0.76	-1.0	2.97 ± 0.024
	6.0	99.40	1.28	-0.60	5.964 ±0.08
	9.0	99.80	1.93	-0.20	8.982 ± 0.182
Indigocarmine	2.0	99.60	1.07	-0.40	1.992 ± 0.022
	4.0	99.30	1.42	-0.70	3.972 ± 0.059
	8.0	100.70	1.65	0.70	8.056 ± 0.14
			Inter-day		
Amaranth	4.0	99.40	0.51	-0.60	3.976 ± 0.021
	8.0	99.60	0.97	-0.40	7.968 ±0.081
	12	99.10	1.58	-0.90	11.892 ± 0.197
Methylene blue	3.0	99.20	0.82	-0.80	2.976 ± 0.026
	6.0	100.40	1.16	0.40	6.024 ± 0.073
	9.0	100.10	1.40	0.10	9.009 ± 0.132
Indigocarmine	2.0	99.30	0.95	-0.70	1.986 ± 0.02
	4.0	100.60	1.38	0.60	4.024 ± 0.058
	8.0	99.40	1.85	-0.60	7.952 ± 0.154

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

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	3.0	99.50	0.76	-0.50	2.985 ± 0.024
	6.0	99.10	1.05	-0.90	5.946 ± 0.062
	9.0	99.90	1.60	-0.10	8.991 ± 0.151
Methylene blue	4.0	99.00	0.87	-1.00	3.96 ± 0.036
	8.0	99.70	1.06	-0.30	7.976 ±0.089
	12	100.30	1.79	0.30	12.036 ± 0.226
Orange G	2.0	99.30	1.12	-0.70	1.986 ± 0.023
	4.0	100.80	1.30	0.80	4.032 ± 0.055
	8.0	100.70	1.85	0.70	8.056 ± 0.156
			Inter-day		
Amaranth	3.0	99.40	0.83	-0.60	2.982 ± 0.026
	6.0	99.20	1.27	-0.80	5.952± 0.079
	9.0	99.60	1.69	-0.40	7.968 ± 0.141
Methylene blue	4.0	99.20	0.77	-0.80	3.968 ± 0.031
	8.0	100.20	0.98	0.20	8.016 ±0.082
	12	100.50	1.76	0.50	12.06 ± 0.223
Orange G	2.0	99.80	1.04	-0.20	1.996 ± 0.022
	4.0	99.40	1.45	-0.40	3.976 ± 0.061
	8.0	99.70	1.90	-0.30	7.976 ± 0.159

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

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	1.34	0.90	0.84	0.75
	8.0	1.85	1.12	1.40	1.50
	12	2.20	1.90	2.10	2.40
Methylene blue	3.0	1.15	0.92	1.25	0.85
	6.0	1.60	1.80	1.94	1.70
	9.0	2.30	2.15	2.50	2.10
Indigocarmine	2.0	1.14	1.06	0.90	0.80
	4.0	1.70	1.95	1.70	1.80
	8.0	2.20	2.30	2.25	2.45
		TDF			
Amaranth	3.0	0.82	0.95	1.05	1.15
	6.0	1.46	1.29	1.40	1.55
	9.0	1.93	2.05	2.10	1.93
Methylene blue	4.0	1.02	0.78	1.10	0.90
	8.0	1.50	1.42	1.30	1.50
	12	2.20	1.90	2.10	2.40
Orange G	2.0	1.10	0.90	0.85	0.94
	4.0	2.10	1.85	1.70	1.90
	8.0	2.50	2.30	1.85	2.05

[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*

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:

$$\% \operatorname{Recovery} = \frac{[C_F - C_T]}{C_p} x100 \tag{4}$$

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 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	5.958	99.30±0.80	6.036	100.60±0.55	5.934	98.90±0.79
tablets	4.0	4.0	8.056	100.70±1.09	4.88	100.50±1.10	7.936	99.20±1.26
(10 mg)	4.0	6.0	10.12	101.20±1.37	9.91	99.10±1.50	10.04	100.40±1.60
Powerecta	4.0	2.0	6.03	100.50±0.63	6.072	101.20±0.94	5.97	99.50±1.15
tablets	4.0	4.0	8.144	101.80±0.96	8.056	100.70±1.29	8.032	100.40±0.90
(20 mg)	4.0	6.0	10.09	100.90±1.17	9.90	99.00±1.72	9.92	99.20±0.82
Verdenode	4.0	2.0	5.958	99.30±0.72	6.036	100.60±0.65	5.976	99.60±0.79
b	4.0	4.0	8.032	100.40±1.48	7.88	98.50±1.36	7.944	99.30±0.56
tablets (20 mg)	4.0	6.0	9.87	98.70±1.80	9.94	99.40±1.55	10.06	100.60±1.20

Samples	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	5.916	98.60±1.24	6.90	101.50±0.75	6.072	101.20±0.95
tablets	4.0	4.0	8.096	101.20±1.56	7.952	99.40±1.65	7.896	98.70±0.80
(20 mg)	4.0	6.0	9.90	99.00±0.87	10.08	100.80±1.35	10.20	102.00±1.20
Snafi®	4.0	2.0	6.024	100.40±0.69	5.91	98.50±0.84	5.964	99.40±0.68
tablets	4.0	4.0	7.856	98.20±1.35	8.208	102.60±1.30	8.072	100.90±1.50
(20 mg)	4.0	6.0	10.32	103.20±1.15	9.85	98.50±1.57	10.15	101.50±1.80

[a]Average of six determinations

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[62]. Hence, no significant difference between the proposed methods and the reported methods at the 95 % confidence level with respect to accuracy and precision.

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

Samples	Recovery[a] (%) ± SD			
	Proposed Methods			Reported methods [Ref]
	Amaranth	Methylene blue	Indigocarmine	
Levitra tablets (10 mg VARD)	99.45±0.80	100.36±0.47	99.28±0.71	99.92±0.64 [40]
t-value[b]	1.02	1.23	1.49	
F-value[b]	1.56	1.85	1.23	
Powerectatablets (20 mg VARD)	100.21±0.50	99.64±0.38	99.40±0.85	99.90±0.67 [40]

t-value[b]	0.82	0.75	1.03	
F-value[b]	1.79	3.1	1.6	
Verdenodebtablets (20 mg VARD)	99.26±0.90	99.76±0.54	99.30±0.79	99.50±0.72 [40]
t-value[b]	0.46	0.64	0.41	
F-value[b]	1.56	1.77	1.2	
	Amaranth	Methylene blue	Orange G	
Cialis® tablets (20 mg TDF)	100.32±0.32	99.50±0.73	99.20±0.66	99.79±0.56 [47]
t-value[b]	1.83	0.7	1.52	
F-value[b]	3.06	1.69	1.38	
Snafi® tablets (20 mg TDF)	99.48±0.50	99.90±0.69	99.82±0.39	99.60±0.51 [47]
t-value[b]	0.37	0.78	0.77	
F-value[b]	1.04	1.83	1.71	

[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 its tablets using NBS as brominating 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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