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ORIGINAL ARTICLE

Spectrophotometric and spectrofluorimetric methods for the determination of saxagliptin and vildagliptin in bulk and pharmaceutical preparations

Marwa S. Moneeb *

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

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4-Chloro-7-nitrobenzofurazan;
Spectrophotometry;
Spectrofluorimetry

Abstract New simple and sensitive spectrophotometric and spectrofluorimetric methods have been developed and validated for the determination of saxagliptin (SAX) and vildagliptin (VDG) in bulk and pharmaceutical preparations. The spectrophotometric methods were based on derivatization of the investigated drugs with two reagents: 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) and 4-chloro-7-nitrobenzofurazan (NBD-Cl). For further increase in the sensitivity, the D_1 spectra of the reactions products were also recorded. For NQS reaction, Beer's law was obeyed over the ranges of 5–30 and 7–45 μg mL^{-1} for the absorbance readings; and 3–32 and 5–50 μg mL^{-1} for the derivative readings of SAX and VDG, respectively. For NBD-Cl reaction, Beer's law was obeyed over the ranges of 3–20 and 4.5–35 μg mL^{-1} for the absorbance readings; and 1.5–25 and 2.5–40 μg mL^{-1} for the derivative readings of SAX and VDG, respectively. Spectrofluorimetric methods were developed based on the fact that the derivatized investigated drugs with NBD-Cl reagent are highly fluorescent products. The fluorescence concentration plots were linear over the ranges of 0.02–0.25 and 0.03–0.37 μg mL^{-1} for SAX and VDG, respectively. The fluorescence measurement enabled the detection of SAX and VDG at concentrations of about 3 and 7 ng mL^{-1}, respectively. The proposed methods have been validated and successfully applied to the determination of the investigated drugs in their pharmaceutical preparations. The results obtained were statistically compared to those obtained from reference methods.

1. Introduction

Saxagliptin (SAX), (1S,3S,5S)-2-[(2S)-2-Amino-2-(3-hydroxytricyclo [3.3.1.13,7]dec-1-yl)acetyl]-2-azabicyclo [3.1.0] hexane-3-carbonitrile and Vildagliptin (VDG), ((2S)-1-[2-[(3-hydroxy-1-adamantyl)amino]acetyl]pyrrolidine-2-carbonitrile), are two novel oral hypoglycemic drugs of the dipeptidyl-peptidase-4 (DPP-4) inhibitor class.[1,2] DPP-4 inhibitors represent a new therapeutic approach to the treatment of type

* Tel.: +20 34871317; fax: +20 34873273.
E-mail address: marwamoneeb@yahoo.com

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2 diabetes mellitus. They inhibit the inactivation of incretins, resulting in the stimulation of glucose-dependent insulin release and the reduction of glucagon levels.[3] Few analytical methods have been reported for the determination of the two novel gliptins. SAX has been estimated in tablets simultaneously with either metformin[4,5] or pioglitazone[6] using RP-HPLC. A liquid chromatography tandem mass spectrometry method has been reported for the quantitative determination of SAX and its major metabolite in human plasma.[7] VDG has been determined by few RP-LC methods in pharmaceutical preparations[8,9] and in plasma.[10] RP-LC has also been used to estimate VDG simultaneously with metformin in dosage forms.[5,11]

Among the various methods available for the quantitation of drugs, spectrophotometry and spectrofluorimetry continue to be the most convenient analytical techniques, because of their inherent simplicity, low cost and wide availability in most quality control laboratories. Chromatographic methods require highly sophisticated and expensive instruments and solvents that may not be available in some quality control laboratories in the developing countries. Literature review revealed that no spectrofluorimetric method has been reported for the determination of either SAX or VDG. Only two spectrophotometric methods have been reported for the estimation of SAX in pure form and tablet formulation. In the first method, SAX was directly estimated at 208 nm in methanol.[12] The second method was based on colorimetric determination after charge transfer reactions with 2,3-dichloro-5,6dicyano-1,4-benzoquinone (DDQ) and 7,7,8,8,tetracyanoquinodimethane (TCNQ).[13] Only one spectrophotometric method has been described for the determination of VDG in bulk and dosage forms. This method was based on the colorimetric determination after charge transfer reactions with 2,3-dichloro-5, 6dicyano-1,4-benzoquinone (DDQ); 7,7,8,8,tetracyanoquinodimethane (TCNQ) and tetra-chloro-1,4-benzoquinone (p-chloranil).[14]

Due to the poorly absorbing chromophores in the examined drugs and their highly blue shifted λ_{max}, their determination in their dosage forms based on the direct measurement of their absorption for ultraviolet is susceptible to potential interferences from the co-extracted common excipients and/or impurities. The problem is more aggravated if it is required to determine these drugs in biological fluids. Therefore, derivatization is necessary for their determination. So, the aim of the present work is to develop new simple, sensitive and specific color reactions for SAX and VDG and the employment of these reactions in the development of new spectrophotometric and spectrofluorimetric methods for the determination of both drugs in pharmaceutical formulations. These methods can be considered as useful alternatives to reported methods. They can overcome the drawbacks of the existing spectrophotometric methods and offer better sensitivity.

1,2-Naphthoquinone-4-sulfonic acid sodium salt (NQS)[15–17] and 4-chloro-7-nitrobenzofurazan (NBD-Cl)[18–20] have been used as derivatizing reagents in the development of both spectrophotometric and spectrofluorimetric methods for the determination of many pharmaceuticals bearing a primary or a secondary amino group. SAX bearing a primary amino group and VDG having a secondary amino group are potential candidates for the reaction with both NQS and NBD-Cl. Therefore, the present study was devoted to investigate the reaction of SAX and VDG with both reagents, and use these color reactions in the development of simple and rapid spectrophotometric and spectrofluorimetric methods for the determination of both drugs in pharmaceutical preparations.

2. Experimental

2.1. Apparatus

Spectrophotometric measurements were performed using a Specord S600 diode-array spectrophotometer, associated with WinAspect Software version 2.3, Analytik Jena AG, Germany. Spectrofluorimetric measurements were carried out on a Schimadzu (Kyoto, Japan), model RF-1501 version 3.0 spectrofluorophotometer equipped with 1-cm quartz cell and a 150 W xenon lamp. Thermostated water bath (Kotlermann Hanigsen, Germany) and a Schott Gerate pH Meter CG 820 were used.

2.2. Reagents and reference materials

Pharmaceutical grade saxagliptin hydrochloride, certified to contain 99.8% and Onglyza® tablets (batch no: 1E6034B), nominally containing 5.58 mg anhydrous saxagliptin hydrochloride equivalent to 5 mg SAX per tablet, were kindly supplied from Bristol-Myers Squibb Company (Indiana, USA). Pharmaceutical grade VDG, certified to contain 99.6% and Galvus® tablets (batch no: S0421A), nominally containing 50 mg VDG per tablet, were kindly supplied from Novartis Pharma AG Co. (Basle, Switzerland).

A solution of 0.5% (w/v) of 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS; Hopkin and Williams, Chadwell Health, Essex, England) was prepared by dissolving 250 mg in 50 mL distilled water. The solution was freshly prepared and protected from light during use. A solution of 0.2% (w/v) of 4-chloro-7-nitrobenzofurazan (NBD-Cl; Fluka, Buchs, Switzerland) was freshly prepared by dissolving 100 mg in 50 mL methanol. 0.2 M alkaline borate buffer solutions of pH values 8.5, 9, 9.5 and 10 were prepared by mixing 50 mL of 0.2 M aqueous solution of boric acid and potassium chloride (1 L contains 12.37 g of boric acid and 14.91 g of potassium chloride) with the specified volume of 0.2 M NaOH in 200-mL volumetric flask. The required volume was made up with water and the pH is adjusted by pH meter.[21] Hydrochloric acid solution, 4 M, was prepared. Doubly distilled water was used throughout the work. All solvents and materials used throughout this study were of analytical grade.

2.3. Preparation of standard solutions

SAX and VDG stock solutions (1 mg/mL) were prepared separately by dissolving 50 mg of each drug in 50-mL methanol. Stock solutions were protected from light by aluminum foil and kept refrigerated at ~4 °C. With respect to these storage conditions, these solutions were found to be stable for at least 1 month. The stock solutions were further diluted with distilled water (for reactions with NQS) or methanol (for reactions with NBD-Cl) to obtain working solutions in the concentration ranges specified in Table 1.

Table 1 Experimental parameters of the proposed spectrophotometric (absorbance and derivative) and spectrofluorimetric methods.

Parameter	NQS		NBD-Cl	
	SAX	VDG	SAX	VDG
Working standard solutions (μg mL^{-1})	30–320	50–500	15–250	25–400
pH and volume of borate buffer	pH 10 1 mL	pH 9.5 1 mL	pH 9 1 mL	pH 8.5 1 mL
NQS/NBD-Cl (%w/v)	0.5%	0.5%	0.2%	0.2%
Temperature (°C)	25	50	70	70
Reaction time (min)	10	10	15	20
Diluting solvent	Water	Water	Methanol	Methanol
Measuring wavelength λ_{max} (nm)	475	470	470	468
Peak-trough D_1(nm)	429.5–504.5	422.5–497.5	441.5–489.5	438.5–486.5
λ_{em} (λ_{ex}) (nm)			542 (468)	540 (465)

2.4. General recommended procedures

2.4.1. NQS method

2.4.1.1. Spectrophotometric measurement. One milliliter of the standard working solutions of SAX and VDG (over the concentration ranges shown in Table 1) was transferred into separate 10-mL calibrated flasks. One mL of borate buffer of appropriate pH (as indicated in Table 1) was added followed by 1 mL of NQS solution. The reaction solution was mixed well. That of SAX was allowed to proceed for 10 min at room temperature (25 °C). That of VDG was allowed to proceed for the same time at 50 °C in a thermostatically controlled water bath. The reaction mixture was then made up to the required volume with water. The resulting solution was measured at 475 and 470 nm for SAX and VDG, respectively, against the corresponding reagents' blank treated similarly. The drug concentration was calculated from the corresponding regression equation of the calibration graph.

2.4.1.2. Derivative spectrophotometric measurement. Sticking to the abovementioned procedure, the D_1 spectra of the developed colors were recorded and the peak-trough amplitudes at 429.5–504.5 and 422.5–497.5 nm for SAX and VDG, respectively, were computed.

2.4.2. NBD-Cl method

2.4.2.1. Spectrophotometric measurement. One milliliter of the standard working solutions of SAX and VDG (over the concentration ranges shown in Table 1) was transferred into separate 10-mL calibrated flasks. One mL of borate buffer of appropriate pH (as indicated in Table 1) was added followed by 1 mL of NBD-Cl solution. The reaction solution was mixed well and placed in a thermostatically controlled water bath at 70 °C for a fixed time of 15 min (for SAX) and at the same temperature for a fixed time of 20 min (for VDG). The reaction mixture was quenched by cooling under tap water. Then, it was acidified by adding 0.1 mL of 4 M hydrochloric acid solution, and completed to volume with methanol. The absorbance of solutions was measured at 470 and 468 nm for SAX and VDG, respectively, against the corresponding reagents' blank treated similarly. The drug concentration was calculated from the corresponding regression equation of the calibration graph.

2.4.2.2. Derivative spectrophotometric measurement. Sticking to the abovementioned procedure, the D_1 spectra of the developed colors were recorded and the peak-trough amplitudes at 441.5–489.5 and 438.5–486.5 nm for SAX and VDG, respectively, were computed.

2.4.2.3. Spectrofluorimetric measurement. Aliquots of SAX and VDG reaction mixtures prepared for the spectrophotometric measurement were diluted with methanol to reach the concentration ranges specified in Tables 2 and 3. The relative fluorescence intensity (RFI) of the resulting solutions was measured at the appropriate excitation and emission wavelengths (Table 1). The measured RFI was plotted versus the final concentration to obtain the calibration graph. The drug concentration was calculated from the corresponding regression equation of the calibration graph.

2.4.3. Procedure for commercial tablets

Twenty tablets of each formulation were separately weighed. The coats of Onglyza® were removed by carefully rubbing with a clean tissue wetted with methanol. The tablets were separately finely powdered. An accurately weighed amount of each tablet powder equivalent to 100 mg of the corresponding drug was separately transferred into 100-mL calibrated flasks, and dissolved in about 40-mL of methanol. The contents of the flasks were swirled, sonicated for 20 min, and then made up to the required volume with the same solvent. The contents were mixed well and filtered rejecting the first portion of the filtrate. The prepared solutions were diluted quantitatively with distilled water (for reactions with NQS) or methanol (for reactions with NBD-Cl) to obtain suitable concentration for the analysis. Aliquots of the tablets solutions were treated as under the general recommended procedures for the reaction with NQS and NBD-Cl.

2.5. Determination of the stoichiometric ratio of the reaction

The stoichiometric ratio of the formed products was investigated by Job's continuous variation method.[22] Equimolar stock solutions of each drug, NQS and NBD-Cl were prepared in the previously specified solvents. The drug and both reagents were prepared as 3.17×10^{-3} and 3.30×10^{-3} M for SAX and VDG, respectively. Series of 5-mL portions of the stock solutions of the drug and the reagent were made up comprising different complementary proportions (0:10, 1:9, ….…...

Table 2 Analytical parameters for the proposed spectrophotometric and spectrofluorimetric methods for the determination of SAX.

Parameter	NQS		NBD-Cl		
	A	D_1	*A*	D_1	*F*
Linear range μg mL^{-1}	5–30	3–32	3–20	1.5–25	0.02–0.25
Regression parameters					
a	2.0×10^{-3}	1.7×10^{-3}	1.1×10^{-3}	0.7×10^{-3}	−0.82
b	0.0401	0.0534	0.0633	0.0391	3500
r	0.99995	0.99997	0.99996	0.99997	0.99985
S_a	1.1×10^{-3}	0.9×10^{-3}	5.8×10^{-4}	3.4×10^{-4}	0.41
Lower 95%[a]	−0.0011	−0.0008	−0.0005	−0.0002	−1.9598
Upper 95%[a]	0.0051	0.0042	0.0027	0.0016	0.3198
S_b	4.0×10^{-4}	3.5×10^{-4}	5.1×10^{-4}	4.9×10^{-4}	28
$S_{y/x}$	1.4×10^{-3}	1.2×10^{-3}	6.0×10^{-4}	3.5×10^{-4}	0.89
LOD[b]	1.00	0.48	0.56	0.27	0.003
LOQ[b]	3.01	1.45	1.68	0.80	0.01

[a] Lower and upper confidence limits for the intercept at the 95% confidence level.
[b] Concentrations are in μg mL^{-1}.

Table 3 Analytical parameters for the proposed spectrophotometric and spectrofluorimetric methods for the determination of VDG.

Parameter	NQS		NBD-Cl		
	A	D_1	*A*	D_1	*F*
Linear range μg mL^{-1}	7–45	5–50	4.5–35	2.5–40	0.03–0.37
Regression parameters					
a	-1.8×10^{-3}	-1.5×10^{-3}	1.4×10^{-3}	1.0×10^{-3}	0.95
b	0.0286	0.0383	0.04	0.0248	2400
r	0.99993	0.99994	0.99998	0.99999	0.99972
S_a	1.45×10^{-3}	1.10×10^{-3}	1.00×10^{-3}	1.20×10^{-3}	0.44
Lower 95%[a]	−0.0058	−0.0046	−0.0014	−0.0023	−0.2732
Upper 95%[a]	0.0022	0.0016	0.0042	0.0043	2.1732
S_b	2.6×10^{-4}	2.2×10^{-4}	2.8×10^{-4}	2.7×10^{-4}	20
$S_{y/x}$	1.61×10^{-3}	1.15×10^{-3}	1.50×10^{-3}	1.82×10^{-3}	0.93
LOD[b]	1.84	1.03	1.04	0.32	0.007
LOQ[b]	5.52	3.08	3.12	0.95	0.02

[a] Lower and upper confidence limits for the intercept at the 95% confidence level.
[b] Concentrations are in μg mL^{-1}.

9:1, 10:0, inclusive) in 10-mL calibrated flasks containing 1 mL of the appropriate buffer. The solutions were further manipulated as described under the general recommended procedures described above. The absorbance/RFI of each solution was plotted against the drug mole fraction [drug]/[drug] + [reagent].

3. Results and discussion

3.1. Absorption, derivative and fluorescence spectra

The absorption of SAX and VDG was separately recorded against water (Figs. 1 and 2). It was found that the maximum absorption peaks (λ_{max}) of both drugs were 208 and 203 nm and their molar absorptivities (ε) were 8.32×10^3 and 7.81×10^3 L mol^{-1} cm^{-1} for SAX and VDG, respectively. Because of the highly blue shifted λ_{max} of both drugs, their determination in the pharmaceutical formulations based on the direct measurement of their absorption of ultraviolet light is susceptible to potential interferences from the co-extracted common excipients. As well the low ε value could ultimately result in poor sensitivity. Therefore, derivatization of both drugs to more red shifted light absorbing derivatives was necessary. The reaction between both drugs and NQS/NBD-Cl was performed. The absorption spectra of the products were recorded against reagents' blank. For the reaction with NQS, the products were orange-colored exhibiting λ_{max} at 475 and 470 nm for SAX and VDG, respectively (Figs. 1 and 2). For the reaction with NBD-Cl, the products were yellow-colored exhibiting λ_{max} at 470 and 468 nm for SAX and VDG, respectively (Figs. 1 and 2). Obviously, the λ_{max} of the products was highly red shifted. This could ultimately eliminate any potential interference. As well, the sensitivity (ε) was greatly enhanced to be 1.89×10^4 and 3.03×10^4 L mol^{-1} cm^{-1} for SAX; and 1.31×10^4 and 1.87×10^4 L mol^{-1} cm^{-1} for VDG using NQS and NBD-Cl reagents, respectively. Therefore, the spectrophotometric measurements were carried out at 475 and 470 nm for SAX; and 470 and 468 nm for VDG using NQS and NBD-Cl, respectively. For further increase in the sensitivity, the D_1 spectra of the reactions products were also recorded and the peak-trough amplitudes at 429.5–504.5 and

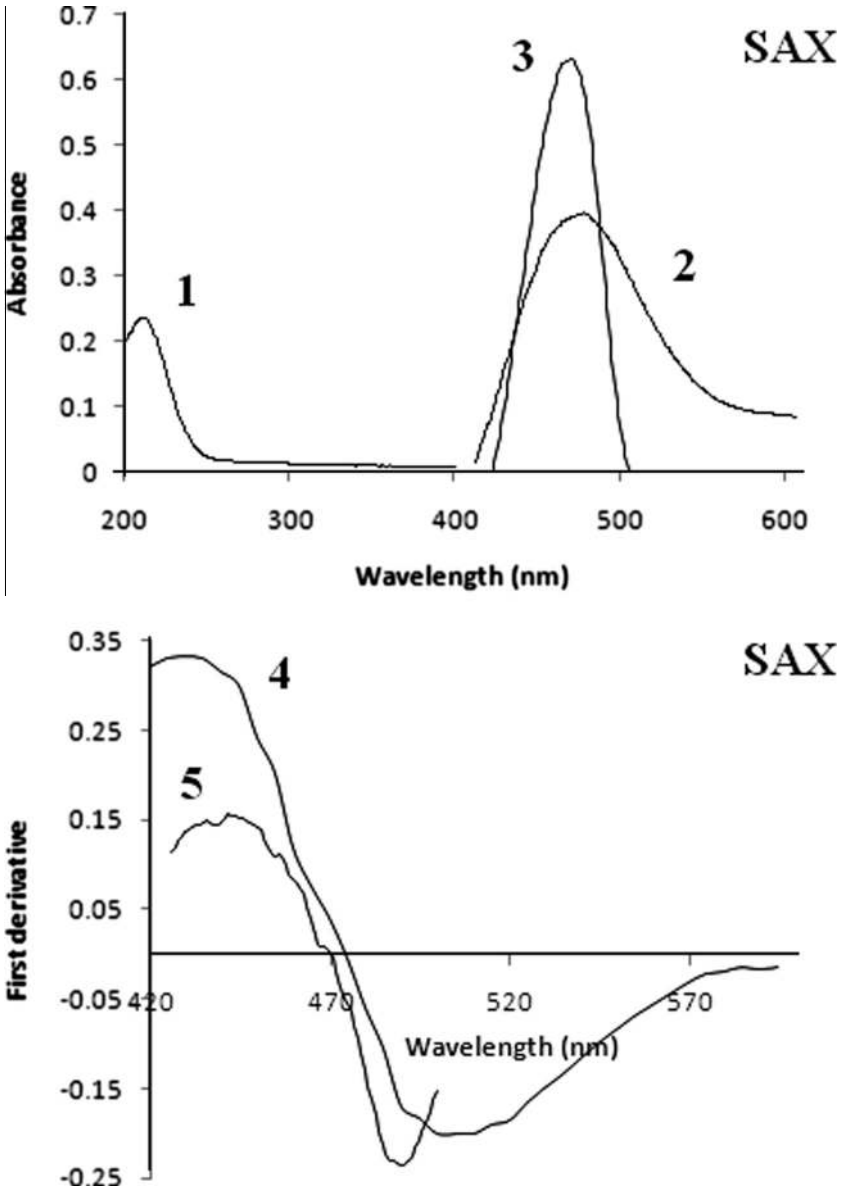

Figure 1 Absorption spectra of 10 μg mL^{-1} SAX against water (1), its reaction product with NQS against reagent blank (2), and its reaction product with NBD-Cl against reagent blank (3). First derivative spectra of its reaction product with NQS (4) and NBD-Cl (5) against reagents blank.

441.5–489.5 nm for SAX; and 422.5–497.5 and 438.5–486.5 nm for VDG using NQS and NBD-Cl, respectively, were measured (Figs. 1 and 2).

SAX and VDG do not have a native fluorescence, thus their derivatization with fluorogenic reagent was necessary for their fluorimetric determination. The reaction between SAX/VDG and NBD-Cl was performed for this purpose. The derivatives exhibited maximum fluorescence intensity at λ_{em} of 542 and 540 nm after their excitation at λ_{ex} of 468 and 465 nm for SAX and VDG, respectively. The excitation and emission spectra of the reaction products of SAX/VDG with NBD-Cl are given in Fig. 3.

3.2. Optimization of reactions variables

Different experimental parameters affecting the color development and its stability were carefully studied and optimized. Such factors were changed individually while keeping others constant. These factors include reagent concentration, pH, temperature, time and diluting solvent.

3.2.1. Effect of reagent concentration

The effect of NQS and NBD-Cl concentrations on their reactions with SAX/VDG revealed that the reactions were dependent on the reagent concentration. It was found that for both SAX and VDG, increasing the reagent concentration increased the color intensity, up to 0.3% and 0.1% (w/v) for NQS and NBD-Cl, respectively (Fig. 4). Beyond these values, no more increase in absorbance was attained. For high precise values, further experiments of both drugs were carried out using 0.5% and 0.2% (w/v) for NQS and NBD-Cl, respectively.

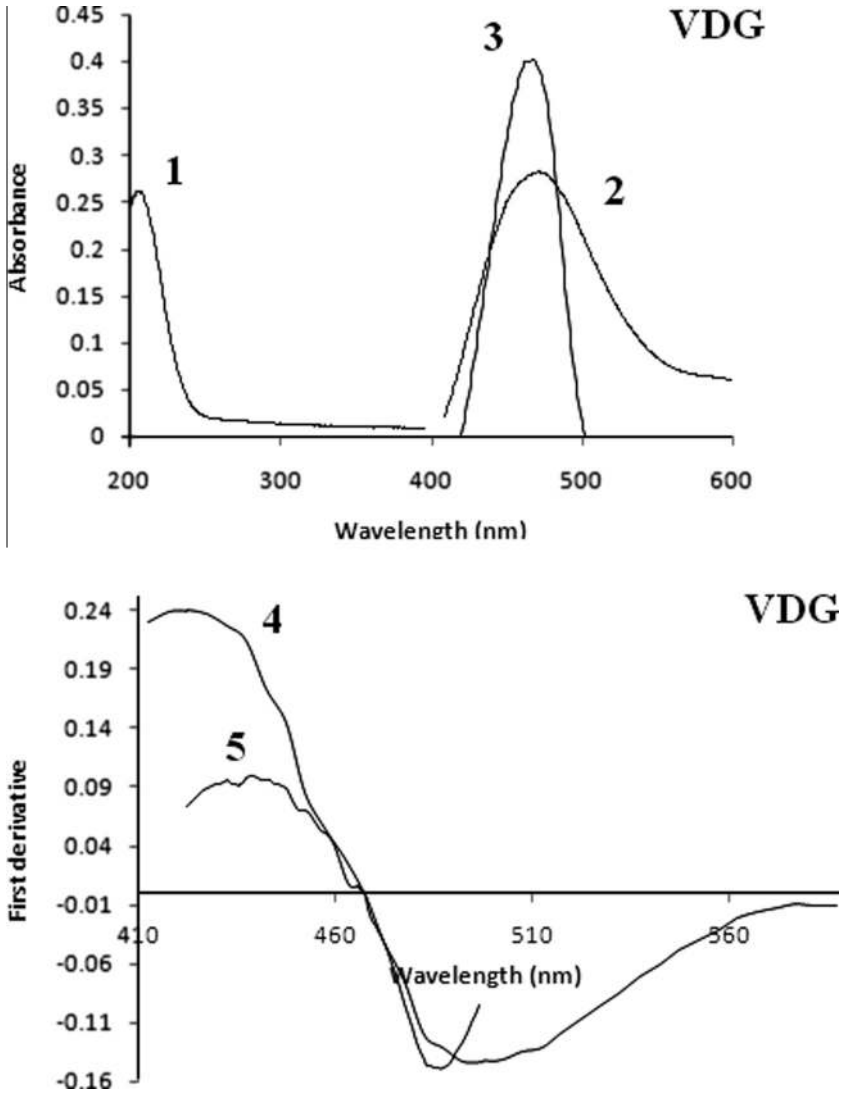

Figure 2 Absorption spectra of 10 μg mL^{-1} VDG against water (1), its reaction product with NQS against reagent blank (2) and its reaction product with NBD-Cl against reagent blank (3). First derivative spectra of its reaction product with NQS (4) and NBD-Cl (5) against reagents blank.

3.2.2. Effect of pH

To generate the nucleophile from SAX/VDG and activate the nucleophilic substitution reaction, alkaline medium was necessary. The influence of pH on the reaction of SAX/VDG with both reagents was studied and optimized to obtain maximum color intensity (Fig. 5). The pH was varied over the pH range of 7–11 using 0.2 M borate buffer. Upon increasing the pH, higher absorbance values were obtained with maximum absorbance at pH values 10 and 9 for SAX; and 9.5 and 8.5 for VDG using NQS and NBD-Cl, respectively. At higher pH values, a sharp decrease in the readings occurred. This was attributed probably to the increase in the concentration of hydroxide ion that holds back the reaction of the investigated drugs with both reagents.[15,16] At pH 0–6, the absorbance of the product is close to 0. The possible reason may be that the amino group is protonized and turned into protonated amine salt. So, it loses nucleophilic capability, and the nucleophilic substitution reaction cannot take place easily.[15] Other buffers having the same pH values such as phosphate buffer and citric acid-phosphate (Mcllvaine's buffer) were tested. Borate buffer was found to be superior because it resulted in more stable highly colored solutions. Also some inorganic bases were tried like sodium hydroxide, borax and disodium hydrogen phosphate, all prepared as aqueous solutions of a concentration range of 0.1–0.5 M. Unfortunately, high blank readings and/or non-reproducible results were observed with them.

3.2.3. Effect of temperature and time

The effect of temperature was studied over the range of 20–90 °C with constant heating time. It was found that the reaction of SAX with NQS was negligibly affected by raising the temperature. Maximum absorbance reading was attained in 10 min at room temperature (25 °C). Longer reaction time up to 30 min did not affect the reaction. Heating the reaction mixture in the range of 40–50 °C had slight positive effect on the reaction. However, to simplify the procedure, further experiments of SAX with NQS reagent were carried out at room temperature (25 °C) for 10 min. The reaction of VDG

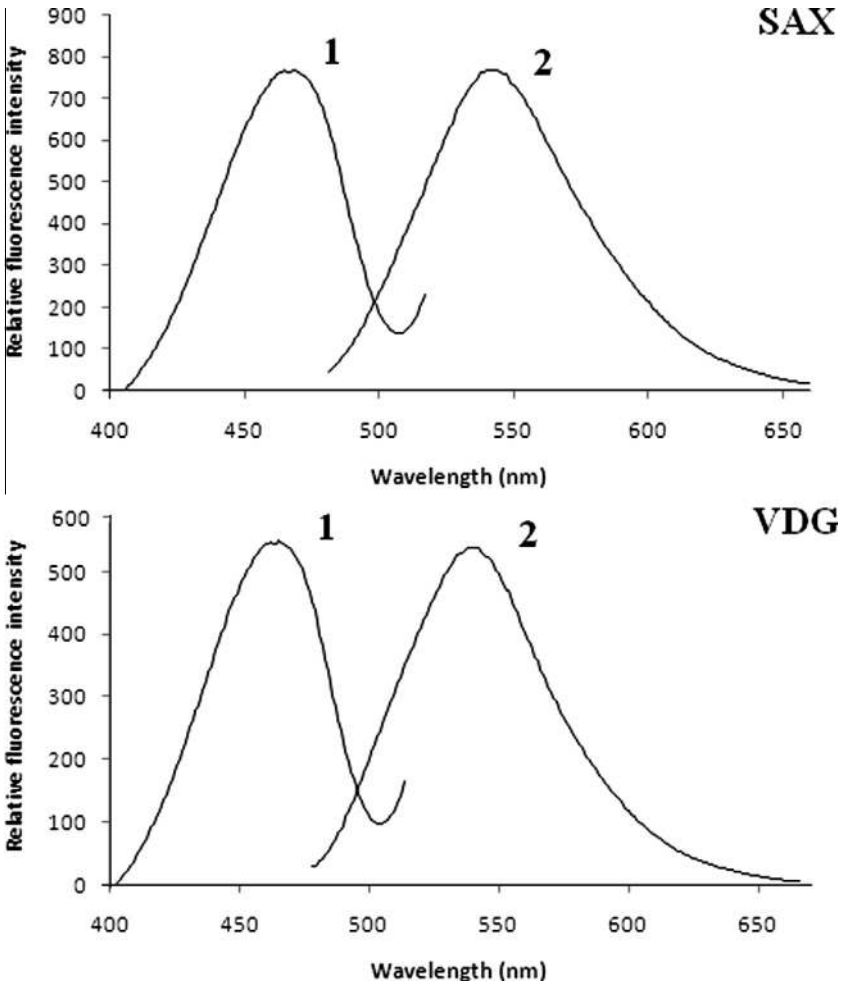

Figure 3 Excitation (1) and emission (2) spectra of the reaction product of (0.2 μg mL^{-1}) SAX and VDG with NBD-Cl in methanol.

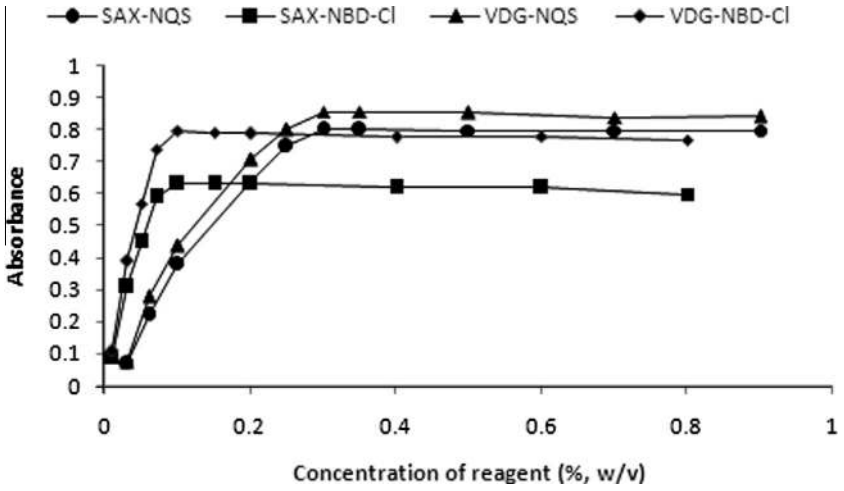

Figure 4 Effect of NQS and NBD-Cl concentrations on their reactions with SAX (20 and 10 μg mL^{-1}) and VDG (30 and 20 μg mL^{-1}), respectively.

with NQS at room temperature was found to be slow, as it required ~40 min for completion. Increasing the temperature of the water bath produced an increase in the product absorbance up to 40 °C. Above which, almost constant absorbance values were obtained. Maximum absorbance reading was attained in 10 min at 40 °C. Longer heating time, up to 30 min, did not

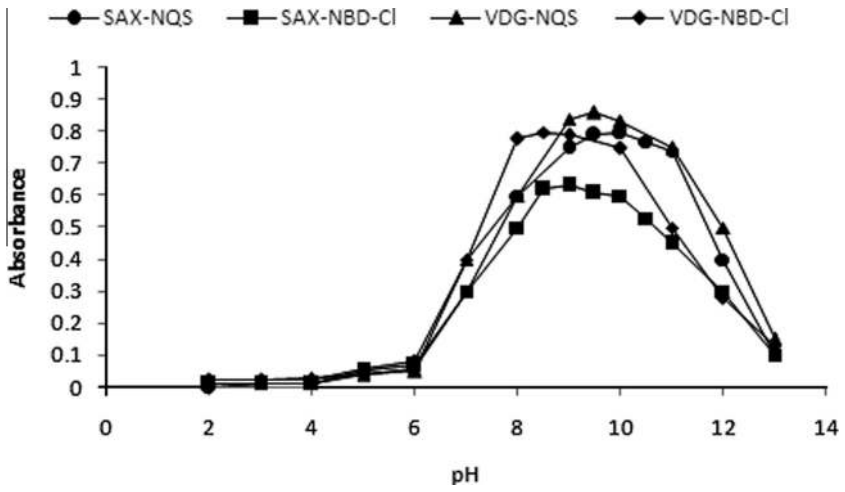

Figure 5 Effect of pH on the reaction of SAX (20 and 10 μg mL^{-1}) and VDG (30 and 20 μg mL^{-1}) with NQS and NBD-Cl, respectively.

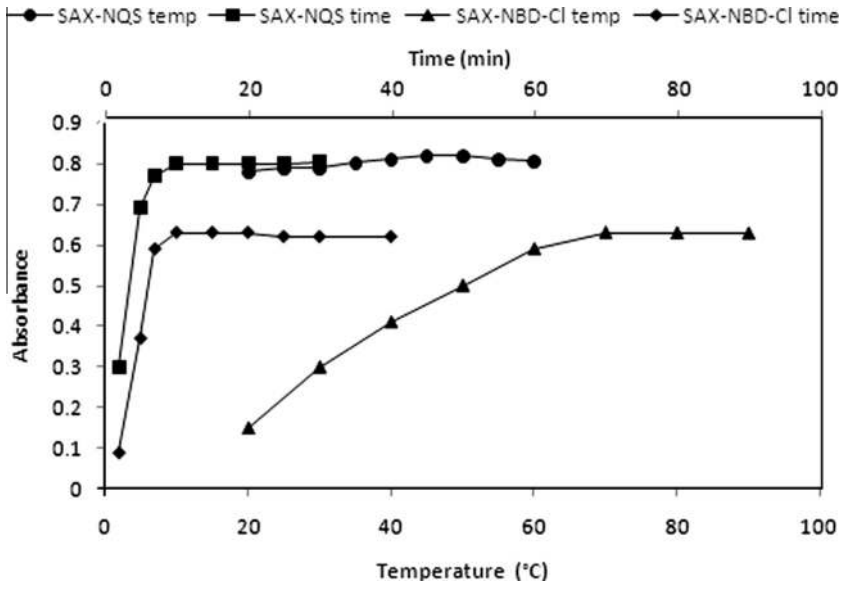

Figure 6 Effect of temperature and time on the reaction of SAX (20 and 10 μg mL^{-1}) with NQS and NBD-Cl, respectively.

affect the reaction. Therefore, for high precise values, further experiments of VDG with NQS reagent were carried out at 50 °C for 10 min.

The reaction of SAX and VDG with NBD-Cl has not been completed at room temperature (25 °C) for at least 1 h. Increasing the temperature of the water bath up to 70 °C produced an increase in the absorbance of the reaction product. Beyond this temperature, almost constant readings were obtained. Proceeding the reactions at 70 °C for varying time intervals revealed that maximum readings were attained by heating at 70 °C for 10 and 15 min for SAX and VDG, respectively. Heating for longer periods up to 40 min had a negligible effect on the obtained readings. So, for high precise values, further experiments of SAX and VDG with NBD-Cl reagent were carried out at 70 °C for 15 and 20 min, respectively (Figs. 6 and 7).

3.2.4. Effect of diluting solvent

Different solvents, such as water, acetone, methanol, ethanol, isopropanol, acetonitrile and 1,4-dioxane were tested as potential diluting media. For NQS reactions, water was found to be the optimum solvent for both drugs, as the highest absorbance values were obtained. For NBD-Cl reactions, the highest readings were obtained when methanol was used for dilution. With NBD-Cl, significantly high absorbance and fluorescence backgrounds were observed. This was attributed to the hydrolysis of NBD-Cl to the corresponding hydroxy derivative, namely, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH).[23] The readings of NBD-OH were found to be quenched by decreasing the pH of the reaction mixture to less than one.[24] Therefore, acidification of the reaction medium prior to dilution and measurement of absorbance or fluorescence was necessary. This resulted in a significant decrease in the reagent blank color

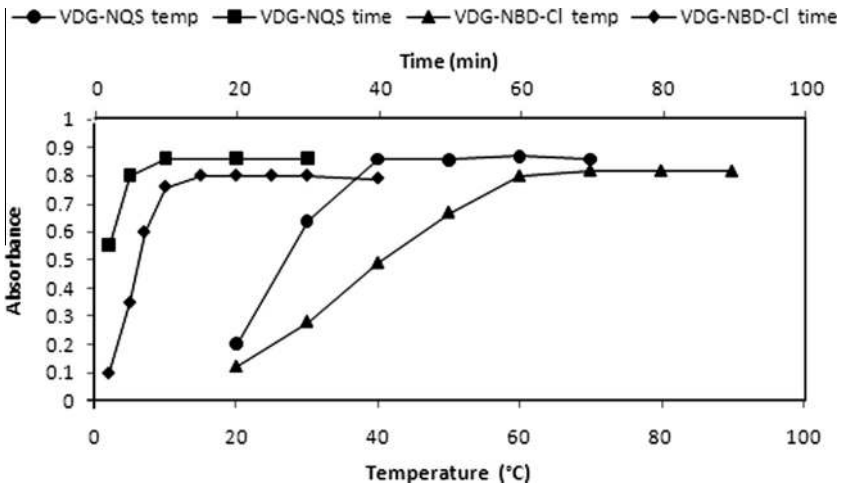

Figure 7 Effect of temperature and time on the reaction of VDG (30 and 20 μg mL^{-1}) with NQS and NBD-Cl, respectively.

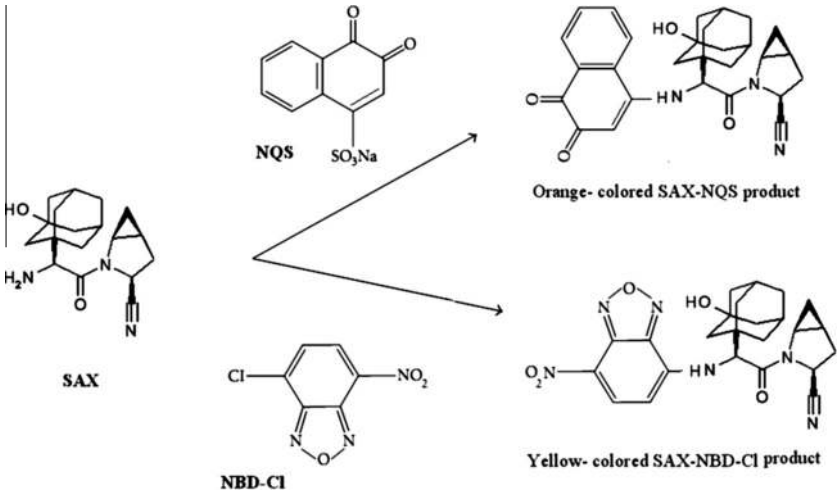

Figure 8 Scheme for the reactions pathways of SAX with NQS and NBD-Cl.

intensity due to the destruction of NBD-OH without affecting the reaction product. Hence, the sensitivity was enhanced. This step was accomplished by the addition of 0.1 mL of 4 M hydrochloric acid solution to the reaction mixtures of both drugs.

3.2.5. Stability of the chromophores and fluorophores

Under the aforementioned optimum conditions, it was found that the absorbance of the chromophores (SAX-NQS, VDG-NQS, SAX-NBD-Cl and VDG-NBD-Cl) and the RFI of the fluorophores (SAX-NBD-Cl and VDG-NBD-Cl) remained stable for at least 4 h at an ambient temperature and at least for 48 h when kept at 4 °C in the dark. This allowed the processing of large batches of samples, and their comfortable measurements with convenience.

3.3. Stoichiometry of the reactions

Under the optimum conditions, the stoichiometry of the reactions between SAX and VDG was investigated by Job's method.[22] The symmetrical bell shape of Job's plot (not shown data) indicated that the drug: reagent ratio was 1:1 for both drugs using both reagents. This ratio was expected as the investigated drugs contain only one center (primary amino group in SAX and secondary amino group in VDG) available for the reaction. Based on this ratio, the reactions' pathways were postulated to be proceeded as shown in Figs. 8 and 9. The primary amino group in SAX and the secondary amino group in VDG exhibit nucleophilic character in alkaline medium due to the availability of the lone pair of electrons on the nitrogen atom. By examining NQS structure, it is clear that C_4 (attached to the sulfonic group) is an electron deficient center due to the presence of C_{3-4} double bond in conjugation with the C_2 carbonyl group. Therefore, through nucleophilic substitution reaction[15], SAX and VDG can react with NQS forming intensely orange-colored products. Both drugs can react with NBD-Cl too forming intensely yellow-colored products. Such reaction proceeds through the nucleophilic substitution of the chloride atom of the reagent.[18]

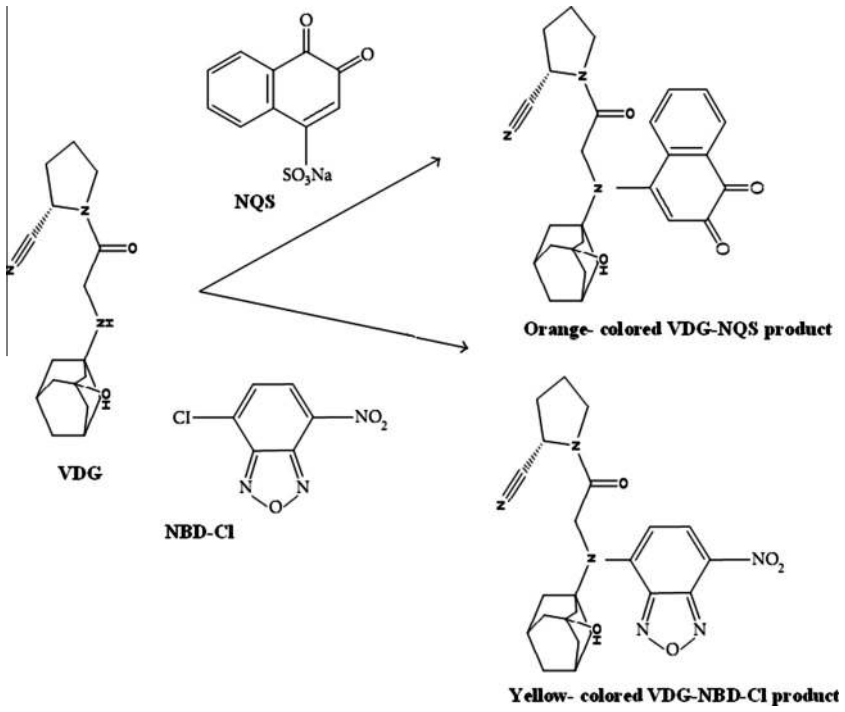

Figure 9 Scheme for the reactions pathways of VDG with NQS and NBD-Cl.

3.4. Validation of the methods

3.4.1. Linearity

For each of the proposed methods, a series of six concentrations of SAX/VDG standard solutions prepared in triplicate was derivatized as previously described. Calibration curves were constructed by plotting the measured response as a function of the corresponding concentration. Linearities for all methods were calculated using the least squares regression model. Regression parameters of the developed assays are summarized in Tables 2 and 3. Other statistical parameters, namely standard deviation of the intercept (S_a), slope (S_b) and the residuals ($S_{y/x}$) were calculated as well (Tables 2 and 3). The good linearity of the calibration graphs and the negligible scatter of the experimental points are clearly evident by the high values of the correlation coefficients, low intercepts and standard deviations. To test if the intercept is differing significantly from zero, its confidence limits at 95% significance level are determined (the confidence limits for the intercept are given by $a \pm t_{(n-2)}s_a$).[25] For all the proposed calibrations, the 95% confidence interval of the intercept includes the ideal value of zero (Tables 2 and 3).

3.4.2. Detection and quantitation limits

In accordance with the recommendations of ICH[26], the limit of detection, LOD = 3.3 σ/s, where σ is the standard deviation of replicate determinations of the blank and s is the sensitivity, namely the slope of the calibration graph. The limit of quantitation, LOQ, is defined as 10 σ/s. The LOD and LOQ of the proposed methods are presented in Tables 2 and 3. Obviously, the LOD and LOQ values are lower for the spectrofluorimetric methods due to the higher sensitivity of this technique.

Table 4 Accuracy and precision of the proposed methods for the determination of SAX.

Method		Accuracy	Precision	
		% Mean recovery[a]	Intra-day RSD %	Inter-day RSD %
NQS	μg mL^{-1}			
A	5	100.3	0.51	0.73
	15	99.4	0.55	0.64
	30	100.8	0.69	0.86
D_1	3	99.7	0.46	0.60
	15	99.9	0.52	0.69
	30	100.4	0.40	0.51
NBD-Cl	μg mL^{-1}			
A	3	99.6	0.64	0.78
	10	100.2	0.57	0.85
	20	100.0	0.43	0.61
D_1	2	100.1	0.39	0.56
	10	99.5	0.48	0.67
	20	99.9	0.54	0.71
F	0.02	99.5	0.74	0.92
	0.10	99.0	0.71	0.96
	0.20	100.3	0.63	0.84

[a] Each result is the mean of five experiments.

3.4.3. Accuracy and precision

Five replicate determinations at three different concentration levels were carried out on the same day and for three consecutive days to test the accuracy, repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed methods. As shown in Tables 4 and 5, the %

Table 5 Accuracy and precision of the proposed methods for the determination of VDG.

Method		Accuracy	Precision	
		% Mean recovery[a]	Intra-day RSD %	Inter-day RSD %
NQS	μg mL^{-1}			
A	7	99.4	0.70	0.91
	20	101.0	0.42	0.73
	40	99.8	0.61	0.84
D_1	5	99.7	0.62	0.80
	25	99.3	0.53	0.82
	50	100.5	0.49	0.67
NBD-Cl	μg mL^{-1}			
A	5	100.2	0.56	0.81
	15	99.0	0.44	0.79
	30	99.5	0.39	0.60
D_1	3	99.6	0.42	0.67
	20	99.9	0.31	0.54
	40	100.7	0.38	0.62
F	0.03	99.9	0.81	1.01
	0.20	100.4	0.86	1.13
	0.30	99.3	0.79	1.25

[a] Each result is the mean of five experiments.

recovery and RSD % values indicate the good accuracy and precision of the methods.

3.4.4. Specificity

The specificity of the methods was evaluated by investigating the interference liabilities from the common excipients that might be added during pharmaceutical formulation. Samples were prepared by mixing known amounts (5 mg SAX/50 mg VDG) with various amounts of the common excipients: starch (50 mg), glucose (10 mg), lactose (10 mg), acacia (10 mg), talc (5 mg) and magnesium stearate (10 mg). These laboratory prepared samples were analyzed by the proposed methods applying the general recommended procedures previously mentioned. Good recoveries of both drugs ranging from 99% to 101% with RSD % ranging from 0.5% to 0.9% were obtained. This confirmed the absence of interference from any of the common excipients with the determination of SAX/VDG by the proposed methods.

3.4.5. Robustness

The robustness of the developed methods was examined by evaluating the influence of small but deliberate variations of the reaction conditions.[21,26] One parameter was changed at a time, whereas all others were kept constant. Variation of the reagent volume by ±0.2 mL, buffer pH by ±0.2 pH units, heating temperature by ±5 °C, reaction time by ±5 min and measurement wavelength by ±2 nm did not significantly affect the developed methods. This provided an indication for the reliability of the proposed methods during their routine application for the analysis of SAX/VDG.

3.5. Assay of pharmaceutical preparations

The proposed methods were applied to the analysis of commercially available tablets. The amount of SAX/VDG was calculated from the regression equation of the calibration curves. Satisfactory results were obtained for each compound in good agreement with the label claim as shown in Table 6. The results were statistically compared to those obtained by the reference methods[13,14] with respect to the accuracy (*t*-test) and precision (*F*-test). The values of the calculated *t* and *F* at 95% confidence level were less than the tabulated ones. This revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the reported ones.

4. Conclusion

The present paper described the successful evaluation of NQS and NBD-Cl as analytical reagents in the development of simple, rapid, selective and sensitive spectrophotometric and spectrofluorimetric methods for the determination of SAX and VDG in bulk and pharmaceutical preparations. In contrast with the previously reported methods, the developed methods in this work have many advantages: they do not need expensive sophisticated apparatus; they are simple and rapid; they

Table 6 Application of the proposed methods to the determination of SAX and VDG in their pharmaceutical preparations.

	NQS		NBD-Cl			Ref.[a]
	A	D_1	*A*	D_1	*F*	
Onglyza®						
Mean found %[b]	100.25	100.29	100.11	100.03	100.40	100.17
±SD	0.65	0.51	0.60	0.49	0.70	1.13
Variance	0.42	0.26	0.36	0.24	0.49	1.28
t (2.31)[c]	0.14	0.22	0.10	0.25	0.39	
F (6.39)[c]	3.02	4.91	3.55	5.32	2.61	
Galvus®						
Mean found %[b]	99.90	100.01	100.22	100.25	99.80	100.13
±SD	0.54	0.49	0.40	0.37	0.69	0.41
Variance	0.29	0.24	0.16	0.14	0.48	0.17
t (2.31)[c]	0.76	0.42	0.73	1.01	1.92	
F (6.39)[c]	1.73	1.43	1.05	1.23	0.35	

[a] Spectrophotometric methods using TCNQ.[13,14]
[b] Values are mean of five determinations.
[c] Figures in parentheses are the tabulated *t* and *F* values at $p = 0.05$.

have high sensitivity; and they are devoid of any potential interference. Furthermore, the used analytical reagents are inexpensive; have excellent shelf life; and are available in any analytical laboratory. Therefore, the proposed methods can readily be applied to routine quality control testing of SAX and VDG. The described spectrofluorimetric methods are the first fluorimetric methods for the analysis of the investigated drugs. Due to the remarkably high sensitivity of these fluorimetric methods, further studies should be directed toward their application to the analysis of the investigated drugs in biological samples.

5. Conflict of interest

None.

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