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Development and validation of a colorimetric method for determination of tadalafil using ion-pair complexation with bromophenol blue

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Abstract
Tadalafil belongs to the class of phosphodiesterase (PDE5) inhibitors used for the management of erectile dysfunction and other conditions. The study reports a colorimetric method for the determination of tadalafil. The method is based on the reaction of the drug with bromophenol blue in acetate buffer (pH 2.8) to form a yellow coloured complex showing maximum absorbance at 415 nm. A linear calibration curve was obtained between 2 and 22 µg/mL of tadalafil with correlation coefficient (r^2) of 0.972 and molar absorptivity of 15,267 $Lmol^{-1}cm^{-1}$. The method similarly demonstrated good precision with intra-day precision ranging from 3.68-5.89 %RSD and inter-day precision of 0.66 – 4.45 % RSD. The developed method was successfully applied to the analysis of tadalafil in tablet formulation and could serve as a useful tool in the quality assessment of this medication.

Keywords: Tadalafil; Bromophenol blue; Ion-pair complexation; Colorimetry

INTRODUCTION

Tadalafil (TDF) is a phosphodiesterase 5 (PDE5) inhibitor used primarily in the management of erectile dysfunction [1]. It has also found use in the treatment of benign prostatic hyperplasia [2] and pulmonary arterial hypertension [3]. It acts by blocking conversion of cyclic guano-sine monophosphate (cGMP) into its inactive form, which results in accumulation of cGMP and subsequent enhancement of nitric oxide mediated penile smooth muscle relaxation [4]. It has the advantage of longer duration of action and less propensity to cause abnormalities of vision as compared to other PDE5 inhibitors such as sildenafil and vardenafil [5] .

Quite a few methods have been employed for the estimation of tadalafil in pharmaceuticals, for example; UV spectroscopy [6–8], HPLC [9,10] Liquid Chromatography-tandem mass spectrometry [11], Gas chromatography-mass spectrometry [12], Spectrofluorimetry [13], Voltametry [14], Capillary electrophoresis [15], Densitometry [16]. A review of major reference literature indicates that Liquid chromatography is one of the official assay methods for the drug [17]. The United States Pharmacopeia [18] similarly reported the use

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of liquid chromatography with a combination of acetonitrile, water and trifluoroacetic acid (35:65:01) for the assay of tadalafil. These assay methods are expensive in terms of instruments, reagents and technical knowhow required to implement them.

A previous survey had shown that drugs used for the management of erectile dysfunction are highly prone to falsification [19]. The use of fake and substandard medications have potentially devastating consequences on human health such as serious adverse drug reactions, treatment failure and even development of drug resistant infections [20]. It has been stated that it can be challenging to implement full monograph testing of medicines in low and middle income countries due to the non-availability of modern and sophisticated analytical equipment [21]. It is therefore important to develop simple, reliable and cost effective methods for evaluating the quality of drugs in circulation. To the best of our knowledge, there is no published report on the colorimetric determination of tadalafil using bromophenol blue.

EXPERIMENTAL METHODS

Chemicals/ reagents. Tadalafil active pharmaceutical ingredient was obtained from Evans Therapeutics Ltd Nigeria. Four (4) different brands of tadalafil tablet formulation obtained from retail Pharmacy outlets in Jos Nigeria and coded A - D. Bromophenol blue (BDH Chemicals Ltd, Poole, England), Absolute Ethanol (Sigma Aldrich, Germany), Glacial acetic acid (Aldrich Ltd, United States of America), Potassium chloride (Merck®, Germany), Sodium Acetate (Sigma Aldrich, Germany), Hydrochloric acid (Sigma Aldrich, Germany)

Equipment. Shimadzu UV-Visible spectrophotometer 1650 model Double beam (Shimadzu, Japan) was used for all absorbance measurements.

Preparation of solutions and reagents. Preparation of tadalafil stock solution. Standard solution of tadalafil was prepared by weighing 0.01g of pure tadalafil API into a 100 mL volumetric flask. To this, 50 mL of ethanol was added, and the entire content was swirled to dissolve the tadalafil. Additional ethanol was then added to make up the volume to mark. Working concentrations of the solutions were subsequently prepared as needed.

Preparation of bromophenol blue solution. A 0.0670 g sample of bromophenol blue (BPB) was weighed and transferred into a 100 mL volumetric flask. Then, 10 mL of ethanol was added to the content to dissolve the BPB. The content was swirled after which distilled water was added to the 100 mL mark.

Preparation of buffer solutions. Hydrochloric acid-Potassium Chloride buffer (pH 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2) was prepared using 0.1 $molL^{-1}$ Potassium chloride and adjusting the pH to the appropriate value using 0.1 $molL^{-1}$ hydrochloric acid. Acetic acid–sodium acetate buffer (2.45, 2.8, 3.4, 3.6 and 4.0) was prepared by mixing 0.2 M acetic acid with 0.2 M sodium acetate to give the suitable pH value. Finally, Phthalate buffer pH (2.2, 2.6, 3.0, 3.4 and 3.8) was prepared using 0.2 mol L^{-1} potassium hydrogen phthalate and 0.2 mol L^{-1} HCl with the pH adjusted to the desired value.

Analytical procedure. A 1 mL portion of tadalafil solution (18 µg/mL) was transferred into a 10 mL volumetric flask with the aid of a pipette. To this, 2 mL of acetate buffer (pH 3.6) was added. Then, 2 mL ($1x10^{-3}$ mol L^{-1}) of bromophenol blue (BPB) was added and mixed gently. The solution was made up to 10 mL with distilled water. The solution was transferred into a separating funnel and extracted with 5 mL of chloroform. The aqueous phase was extracted again with another fresh 5 mL portion of chloroform. The chloroform layers were combined and scanned

between 200 – 750 nm to determine the wavelength of maximum absorbance.

Optimization of reaction conditions. Optimal reaction conditions for the formation of the ion-pair complex were determined by experiments to investigate the effects of type of organic solvent used for extraction, type and pH of buffer used, volume of the ion-pairing reagent used, shaking time and temperature on formation of the ion-pair complex.

Effect of the extraction solvent. The effect of several organic solvents on extraction of the ion-pair complex was investigated. Chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), ethyl acetate ($C_4H_8O_2$), diethyl ether $(C_2H_5)_2O$ and dichloromethane (CH_2Cl_2) were evaluated for effective extraction of the coloured ion pair complex from the aqueous phase. After extraction of the complex, the absorbance was measured at the previously determined maximum of 415 nm against a reagent blank prepared in a similar manner but excluding the drug. All solutions were prepared in triplicates and average absorbance taken for each.

Effect of type and pH of buffer. Three different buffers of varying pH ranges were evaluated in the study as part of the method optimization. Specifically, the analytical procedure was repeated using KCl-HCl buffer (pH 1.2, 1.4, 1.6, 1.80, 2.0 and 2.2), Acetate buffer (pH 2.45, 2.80, 3.40, 3.60 and 4.00) as well Potassium hydrogen phthalate – hydrochloric acid buffer (pH 2.2, 2.6, 3.0, 3.4 and 3.8). Each time, the corresponding absorbance value at the various pH values tested was taken against reagent blank at 415 nm.

Effect of shaking time. The effect of shaking time on ion pair complexation was evaluated by conducting experiments in which the analytical procedure was repeated while ranging the shaking time between 0.5 minutes to 4.0 minutes. The other reaction conditions were kept constant. The absorbance of the extracted complex was taken at 415 nm against a reagent blank.

Effect of temperature. The effect of temperature on the reaction was investigated by repeating the experiment at various temperatures of 25 ^{o}C, 30 ^{o}C, 35 ^{o}C and 40 ^{o}C. In each case, the solutions were prepared in triplicates and absorbance values measured at 415 nm against a reagent blank.

Effects of bromophenol blue concentration ($1.0x10^{-3}$ M). The effect of BPB concentration on complex formation was evaluated by repeating the procedure but varying the BPB volume (0.8, 1.2, 1.6, 2.0 and 2.4 mL). In each case, solutions were prepared in triplicates and their corresponding absorbances were taken at 415 nm against a reagent blank.

Effect of sequence of mixing. The effect of sequence of mixing of the reagents was investigated by repeating the analytical procedure but changing the order of addition of the reagents. The changed sequence was TDF (1mL) plus BPB (2 mL) plus 2 mL of acetate buffer (pH 2.8). The solution was shaken and double extraction performed with chloroform as before. The absorbance of the combined chloroform ion pair extract was then measured at 415 nm against a reagent blank.

Stoichiometric relationship. The stoichiometric relationship between tadalafil (TDF) and bromophenol blue (BPB) was investigated using the Jobs method of continuous variation. In this procedure, equimolar concentration of tadalafil ($5.0x10^{-4}$ mol L^{-1}) and bromophenol blue ($5.0x10^{-4}$ mol L^{-1}) were prepared. A series of 10 mL volumetric flasks were then setup and the prepared solution of TDF ($5.0x10^{-4}$ mol L^{-1}) and BPB ($5.0 x10^{-4}$ mol L^{-1}) were added in the ratio; 0.0:2.0, 0.2:1.8, 0.4:1.6, 0.6:1.4, 0.8:1.2, 1.0:1.0, 1.2:0.8, 1.4:0.6, 1.6:0.4, 1.8:0.2 and 2.0:0 respectively. Acetate buffer (pH 2.8) was added and the solution made up to 10 mL mark with distilled water. The resultant solution was extracted twice with chloroform (5 mL) and

the corresponding absorbance of the combined chloroform layer taken against reagent blank at 415 nm.

Stability of the complex. The stability of the ion pair complex formed from the reaction of tadalafil and bromophenol blue under the optimized reaction conditions was monitored over a 48 hour period.

Preparation of the calibration curve. A series of 10 mL volumetric flasks were prepared and TDF solution of concentrations; 2.0 µg/mL, 6.0 µg/mL, 10.0 µg/mL, 14 µg/mL, 16.0 µg/mL, and 22.0 µg/mL transferred into the volumetric flasks respectively in triplicate. To each, 2 mL of bromophenol blue ($1x10^{-3}$ mol L^{-1}) solution was added. Then, 2 mL of acetate buffer (pH 2.8) was also added to each volumetric flask and shaken then made up to the 10 mL mark with distilled water. The solution was then extracted with chloroform (5 mL x 2), the organic layers combined and the absorbance taken at 415 nm against a reagent blank. The above method was repeated on five different days at one experiment per day. The equation for the calibration curve was obtained using the least squares method of linear regression analysis.

Method validation.

Determination of linearity and sensitivity. The linearity was determined from the correlation coefficient (r^2) while the **sensitivity** was determined from the slope of the regression line of the calibration curve. The acceptance criterion for this parameter is a correlation coefficient ≥ 0.95 for the range of 80 to 120% of the target concentration [22].

Determination of precision

Intra day and inter day experiments. Three 10 mL volumetric flasks were washed and prepared. From the tadalafil stock solution 6 µg/mL, 12 µg/mL and 18 µg/mL, were pipetted separately and transferred separately into each of the volumetric flask. Bromophenol blue reagent 2.0 mL ($1x10^{-3}$ mol L^{-1}) was added separately into each of the volumetric flask. After which, 2.0 mL of acetate buffer pH 2.8 was added, and double extraction were done (per each content of the volumetric flask) with 10 mL chloroform after the solution was shaken for 3 minutes. The collected organic layers were separately spiked into blank and their absorbances were taken at 415 nm against a reagent blank. This experiment was repeated five times within a day (intraday) to obtain intra day readings. It was also repeated three times on three different days to obtain inter day precision which is given as percentage relative standard deviation as shown below:

% Relative Standard Deviation (RSD) = S * 100/x

Relative standard deviation is the standard deviation as a fraction of the mean, i.e. S/x. It is usually multiplied by 100 and expressed as a percent relative standard deviation. With regards to the acceptance criteria, ICH guidelines state that the typical RSD should be less than 2% [22].

Determination of Limit of Detection (LOD) and Limit of quantification (LOQ). Limit of detection (LOD) was calculated using the equation LOD = 3 x Sa / slope while Limit of quantitation (LOQ) was determined using the formula:

LOQ = 10 x Sa/ slope

Where Sa = Standard deviation of intercepts of calibration curves.

The acceptance criteria for the quantitation limit is the best estimate of a low concentration that gives an RSD of approximately 10% for a minimum of six replicate determinations or alternatively a signal-to-Noise ratio of 10:1 while the acceptance criteria for the Limit of detection is >2 times base line or alternatively a signal-to-Noise ratio of 3:1 [22].

Determination of molar absorptivity. This was done using the equation for Beer's Law:

$$A = \varepsilon bc$$

Where: A is absorbance (no units); ε is the molar absorptivity with units of L mol^{-1} cm^{-1}; b is the path length of the sample, usually expressed in cm; c is the

concentration of the compound in solution, expressed in mol L^{-1}

Application of developed method to analysis of tablet dosage formulation. The developed method was applied to the analysis of four (4) brands of tadalafil in the market. In the procedure, twenty tablets (brand A, 20 mg TDF per tablet) were weighed individually and total average weight was obtained. The weighed tablets were powdered and an amount of the powdered drug equivalent to 20 mg TDF was transferred into a 20 mL volumetric flask. A 10 mL portion of ethanol was added and the flask was shaken and made up to the 20 mL mark with ethanol. The solution was then filtered through Whatman filter paper No. 41. The solution obtained was further diluted to give a final concentration of TDF of 100 µg/mL. A 1 mL aliquot of the solution above was transferred into a 10 mL volumetric flask, 2 mL of $1x10^{-3}$ $molL^{-1}$ bromphenol blue added, shaken and 2 mL of acetate buffer (pH 2.8) added and the volume made to the 10 mL mark with distilled water. The solution was extracted twice with chloroform (5 mL x 2). The absorbance of the combined extract was then taken against a reagent blank at 415 nm. The other brands of tadalafil coded brand B, C and D were similarly analyzed. All these analyses were performed in triplicates.

RESULTS

Optimum reaction conditions for complex formation

Effect of extraction solvent. Chloroform was found to be the most suitable solvent for extraction of the coloured complex through double extraction with two 5 mL portions, because of its higher efficiency on colour intensity, selective extraction of the ion-pair complex from the aqueous phase, the highest absorbance obtained (Figure 2) and the shortest time to reach the equilibrium between both chloroform and aqueous phases.

Effect of type and pH of buffer. It was noticed that the maximum color intensity and highest absorbance value were observed in an acetate buffer of pH 2.8, which is the optimum pH value for buffers using bromophenol blue (Figure 3). In addition, the stability of the color was enhanced without affecting the absorbance at the optimum pH values. Furthermore, 2.0 mL of acetate buffer gave maximum absorbance and reproducible results.

Effect of shaking time and temperature. The optimum reaction time was investigated from 0.5 to 4.0 min by following the colour development at ambient temperature (25°C). Complete colour intensity was attained after 3.0 min of mixing for all complexes. Raising the temperature up to 30°C had no effect on the absorbance of the formed complexes, whereas above 30°C, the absorbance started to decay.

The absorbance remained stable for at least 48 hours.

Effect of reagent concentration (bromophenol blue $1x10^{-3}$ M). The maximum colour intensity of the complex was achieved with 2.0 mL of bromophenol blue the reagent solutions (1.0×10^{-3} mol L^{-1}). However, a larger volume of the reagent had no pronounced effect on the absorbance of the formed ion-pair complexes as depicted in Figure 4.

Effect of sequence of mixing. The most favorable sequence was (drug–dye–buffer-solvent), for the highest color intensity and the shortest time for developing maximum absorbance, while the other sequences required longer time and produced lower absorbance values.

Stoichiometric relationship. The result of the molar ratio of the reagents (drug: dye) in the ion-pair complexes determined by the continuous variations method (Job's method), indicated that 1:1 (drug: dye) ion-pair complexes was formed at 415 nm (Figure 5).

Calibration curve. Under the optimized reaction conditions, a linear calibration curve was obtained as shown in Figure 6.

Method validation results. The linearity "r" was obtained as **0.972**, and the sensitivity obtained as **0.026**. In addition, molar absorptivity was determined as 15,267 $LMol^{-1}cm^{-1}$ while the LOD and LOQ were determined as 0.475 and 1.586 µg/mL respectively. Furthermore, the results of the precision and recovery determinations are presented in Table 1 and 2 for intra-day and inter-day experiments respectively.

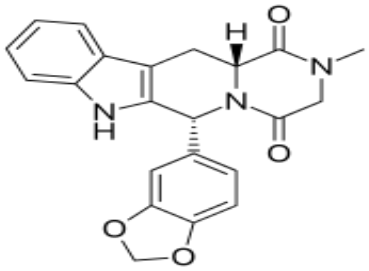

Figure 1: Structure of Tadalafil

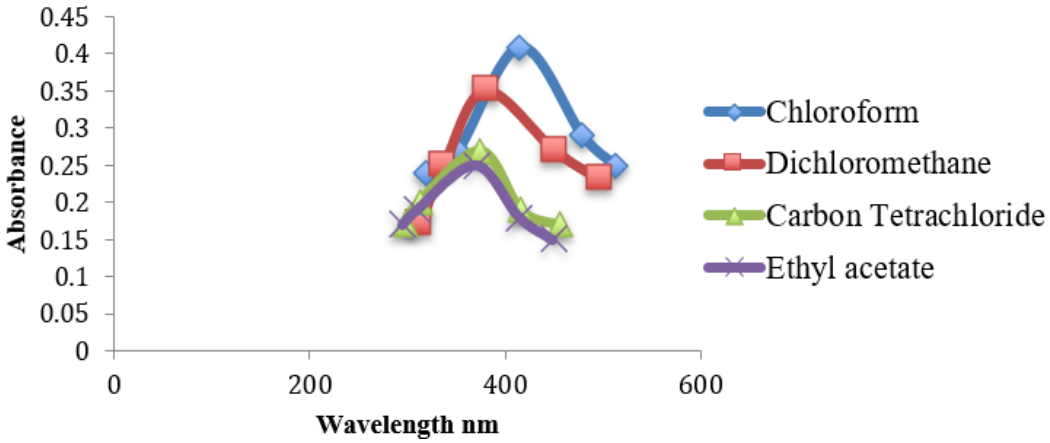

Figure 2: Effect of solvent of extraction on absorbance of the ion-pair complex.

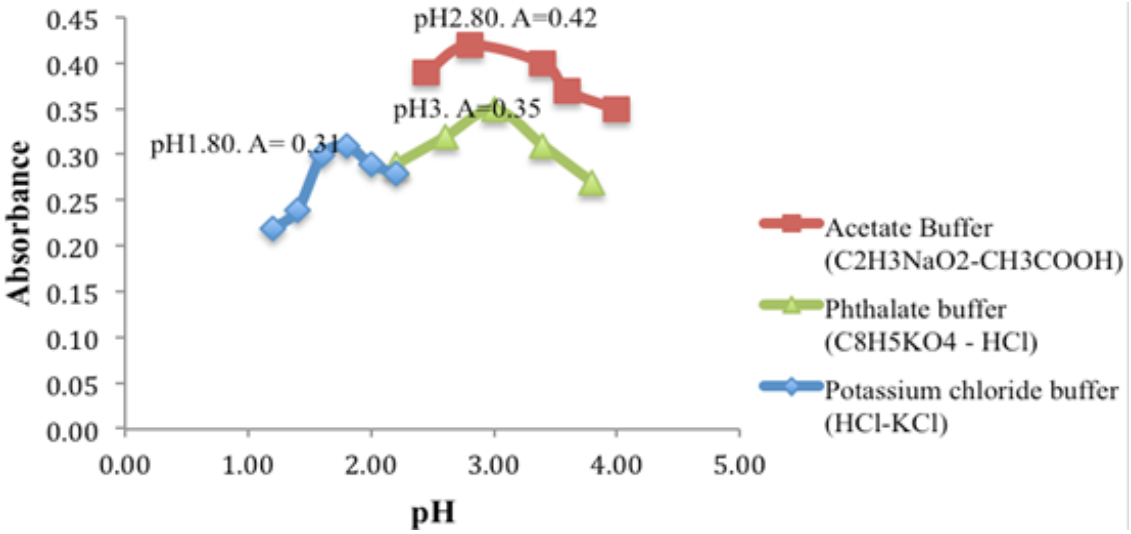

Figure 3: Effect of the pH of the acetate buffer, potassium chloride buffer and phthalate buffer on absorbance of the complex.

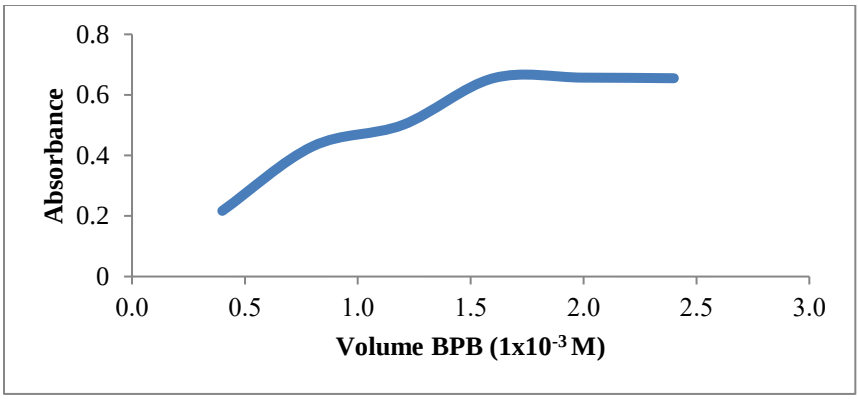

Figure 4: Effect of BPB reagent ($1x10^{-3}$ $molL^{-1}$) on ion – pair complex formation**.**

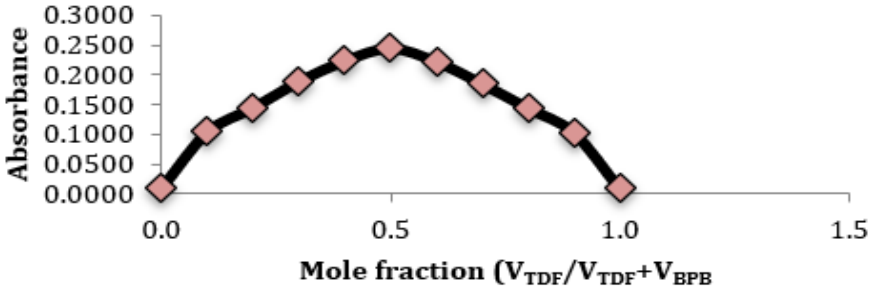

Figure 5: Job's method of continuous variation plot for the reaction of tadalafil with bromophenol blue. [Drug] = [Dye] = $5.0x10^{-4}$ mol L^{-1}

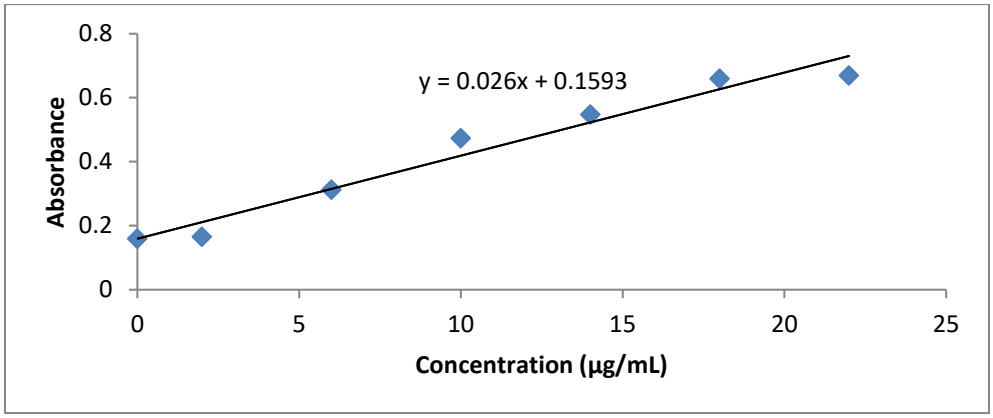

Figure 6: Calibration curve for tadalafil – BPB ion pair complex

Table 1: Intra-day Precision repeatability

Method	Added µg/mL	Recovery %	Precision RSD %
BPB	6.0	94.57	4.44
	12.0	115.60	3.68
	18.0	94.15	5.89

Table 2: Inter-day Precision reproducibility

Added µg/mL	Recovery %	Precision RSD %
6.0	93.70	4.45
12.0	116.45	3.86
18.0	94.64	0.66

Table 3: Optimized Reaction Conditions for ion-pair complexation

Parameters	Optimum Condition Obtained
Maximum Wavelength (λ_{max})	415 nm
Buffer	Acetate buffer pH 2.8
Extraction Solvent	Chloroform (5 mL x 2)
Shaking Time	3 minutes
BPB concentration	2.0 mL (1 x 10^{-3} M)
Stoichiometric relationship	1:1
Sequence of mixing	TDF + BPB + buffer + solvent
Temperature	25 ^{o}C

Table 4: Determination of tadalafil in branded tablet formulations using developed method

Brand	Brand A	Brand B	Brand C	Brand D
% Content	101.4±1.99	109.9±0.72	99.9±1.53	103.7±1.36

DISCUSSION

Generally, a number of drugs and other organic compounds containing basic amino functional groups have been quantitatively determined by taking advantage of their reaction with anionic acidic dyes to form coloured ion pair complexes. In this vein, reaction of tadalafil with bromophenol blue in an acidic buffer led to formation of a yellow coloured solvent extractible ion-pair complex showing maximum absorption at 415 nm. The absorbance of this complex was found to vary in a linear manner with tadalafil concentration and a linear calibration curve was obtained in the concentration range of 2 to 22 μg/mL of tadalafil with correlation coefficient (r^2) of 0.972. This range is wider than the 2-14 μg/mL linearity range obtained in another study in which a colorimetric method for tadalafil was developed using bromocresol purple and methyl orange [23]. The molar absorptivity of the ion pair complex in the present study was determined as 15,267 $Lmol^{-1}cm^{-1}$ implying that the complex showed more intense absorption compared to the species obtained when tadalafil was condensed with isatin to form a bluish-green chromogen with molar absorptivity of 7,700 $Lmol^{-1}cm^{-1}$ in an earlier study [24]. Furthermore, the detection limit of the newly developed ion pair method (0.475 μg/mL) compares favourably against the LOD obtained for another developed colorimetric method for the drug that involved its oxidative reaction with Cerium IV/ Indigo Carmine which had an LOD of 3.5 μg/mL [25].

The acetate buffer (pH 2.8) used in this study ensured that the nitrogenous drug tadalafil was present in its protonated (positively charged) form and the attraction between this species and the bromophenol blue present in its anionic (negatively charged) form drove the complexation reaction forward. The proposed structure of the complex formed is elucidated under reaction mechanism (Scheme 1).

The results of the study further indicated that 1:1 (drug: dye) ion-pair complexes were formed due to electrostatic attraction between the positively charged protonated TDF^+ and negatively charged BPB^- The extraction equilibrium can be represented as follows:

$$TDF^{+}_{(aq)} + BPB^{-}_{(aq)} \rightleftharpoons TDF^{+}\,BPB^{-}_{(aq)} \rightleftharpoons TDF^{+}\,BPB^{-}_{(org)}$$

Where TDF^+ and BPB^- represents the protonated drug and the anion of the dye respectively and the subscript (aq) and (org) refers to the aqueous and organic phases. See reaction mechanism.

The precision experiments (inter day and intraday) conducted as part of the method validation showed that the method is both repeatable and reproducible though some of the %RSD values were slightly above the ICH acceptance criteria of 2% RSD implying that the method may require additional fine-tuning

to further improve its precision. The other validation parameters obtained during the study indicate that the method is accurate and sensitive enough for application in forensic analysis and also for use in routine quality assessment of the drug. In addition, the proposed method is relatively simple, rapid and gave excellent recoveries when used for the determination of tadalafil in commercial tablet formulation. The method is free from the tedious experimental steps, such as heating, that are employed in some of the previously reported methods [26]. Other attractive features of this method are its relative freedom from interference by the usual diluents and excipients normally found in pharmaceutical formulations and the fact that it requires relatively inexpensive equipment (i.e. a colorimeter) to carry out. This is unlike the official assay method for the drug which is liquid chromatography as specified by both the British and United States Pharmacopoeias which requires the use of a HPLC that may not be readily available in quality control laboratories in developing countries.

Conclusion. The study has successfully developed a simple, precise and accurate colorimetric method for the estimation / assay of tadalafil in API (active pharmaceutical ingredients) and formulation. The method was applied for estimation of TDF in four brands of tablet dosage formulations and produced accurate results with no interference from excipients in the formulation. This method can readily be adapted for use in low and middle income countries where sophisticated analytical equipment may not be readily available.

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