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Research Article

SPECTROPHOTOMETRIC AND SPECTROFLUORIMETRIC DETERMINATION OF PREGABALIN VIA CONDENSATION REACTIONS IN PURE FORM AND IN CAPSULES

Hanaa M. Saleh, Magda M. EL-Henawee, Gamal H. Ragab and Omnia F. Mohamed*

Department of Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

ABSTRACT

Three simple, accurate and precise colorimetric methods for the determination of pregabalin in capsules are developed. The first method is based on the reaction of pregabalin with vanillin (Duquenois reagent) in the presence of McIlvain buffer pH 7.5 and the color developed was measured at 394 nm. The linearity range was found to be 20-180 µg ml-1.The second and third mehods are based on condensation of primary amine group of pregabalin with acetylacetone and formaldehyde in presence of acetate buffer pH 5 (Hantzsch reaction) producing a yellow coloured product, which is measured spectrophotometrically at 333 nm or fluorimetrically at 470 nm. Beer's law was valid within a concentration rang 20-160 µg ml-1 spectrophotometrically and 0.2 - 3 µg ml-1 fluorimetrically. All the variables were studied to optimize the reaction conditions. No interference was observed in the presence of common pharmaceutical excipients. The validity of these methods was tested by analyzing pregabalin in its pharmaceutical preparations. Good recoveries were obtained and the results were comparable with those obtained by reported method.

Keywords: pregabalin, spectrophotometry, spectrofluorimetry, Hantzsch, Duquenois reagent.

INTRODUCTION

Pregabalin (PG) Figure 1, (S)-3-(amino methyl)-5-methylhexanoi acid is lipophilic GABA (g-aminobutyric acid) analog[1]. It was approved in the year 2007 for adjunctive treatment of partial seizures in adults[2,3] in United States and Europe, and for the treatment of neuropathic pain from post-therapeutic neuralgia and diabetic neuropathy.

Currently, there is no official analytical procedure for pregabalin in any pharmacopeia but the wide use of this drug has prompted many researches to develop different analytical methods for its determination. Reports for pregabalin determination based on chromatographic methods as, HPLC[4,5] ,gas chromatography-mass spectrophotometry (GC-MS), LC-MS-MS[6,7]. Capillary electrophoresis and nuclear magnetic resonance technique was reported for PG[8]. Spectrophotometric[9-19] and spectroflurometric[20-22]. The chromatographic methods require high cost solvents in addition to tedious pretreatment, Regarding spectrophotometric methods for determination of PG, some of them don't offer high sensitivity[14,16,17] or need tedious extraction procedures[12].Some mehods recommended measurment of absorbance near UV region where interference propably occur[9,10] or use non specific reagent Potassium iodide/potassium iodate[15]. UV-Visible spectrophotometry is the technique of choice in research laboratories due to its low cost and inherent simplicity. This paper reports simple, sensitive and accurate spectrophotometric and spectrofluorimetric methods for determination of

PG. The first method was based on the reaction of PG with Duquenois reagent in the presence of McIlvain buffer pH 7.5 and the second and third are based on reaction of PG with acetylacetone and formaldehyde in presence of acetate buffer pH 5 ,Hantzsch reaction.

The main advantages of the proposed methods are being simple, rapid and not require tedious extraction procedure. Compared to other reported spectrophotometric methods, the proposed methods are either more sensitive or even having comparable sensitivity.

MATERIAL AND METHOD

Instrumentation

-A Shimadzu recording spectrophotometer UV 1800 equipped with 10 mm matched quartz cells was employed for all absorbance measurements

- A Shimadzu RF-5301PC Spectrofluorophotometer equipped with xenon lamp and 10 mm matched quartz cells.

-A Jenway digital pH Meter calibrated with standard buffers was used for checking the pH of the buffer solutions used.

Chemicals

All reagents were of analytical grade and double distilled water was used:

1. pregabalin pure drug and Lyrica® capsules (labeled to contain 75 mg PG per capsule) were obtained from Delta pharm,Egypt.
2. McIlvain buffer was prepared by mixing 35.5 ml of 0.2 M disodium hydrogen phosphate with 64.5 ml of 0.1 M citric acid and pH was adjusted to 7.5 with 0.1 N sodium hydroxide.
3. Duquenois reagent was prepared by mixing 2g of vanillin with 0.3 ml of acetaldehyde and the volume was completed to 50 ml with ethyl alcohol. The reagent is stored in dark.
4. Acetylacetone (El-Nasr Chemical Co., Egypt), 8.4% v/v freshly prepared by mixing 2.1ml acetylacetone with 10 ml acetate buffer pH 4.9 and dilution to 25 ml with distilled water.
5. Formaldehyde (34-40%) (El-Nasr Chemical Co.,), 20% v/v prepared by mixing 5 ml formaldehyde with distilled water to 25 ml.
6. Acetate buffer pH5: Dissolve 13.6 g of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1000 ml.

Standard solution

Solution of 1.0 mg ml^{-1} was prepared by dissolving 100 mg PG in 100 ml distilled water. This solution was further diluted with the same solvent as appropriate to obtain the working concentration range. The stock solution is stable for 7 days when kept in the refrigerator

General procedure

Method 1 (using Duquenois reagent)

Into 10 ml measuring flasks, different aliquots of drug solution were transferred to provide final concentration range 20- 180 µg ml^{-1}. To each flask, 2 ml of Duquenois reagent and 2 ml of McIlvain buffer of pH 7.5 were successively added and set aside at room temperature for 40 min. The volume was made up to the mark with distilled water and the absorbance was measured against a reagent blank at 394 nm. The calibration graph was prepared by plotting absorbance vs. concentration of pregablin.

Method 2 (Spectrophotometric method)

To different aliquots of standard solution containing (20-160) µg pregabalin , 1 ml of 8.4% v/v acetylacetone solution and 0.2 ml of 20% v/v formaldehyde reagents were added in series of 10 ml test tubes . The mixture was heated in a boiling water bath for 7 minutes. Then, cooled and diluted to 10 ml with distilled water. Absorbance was measured at 333 nm against a reagent blank treated similarly.

Method 3 (spectrofluorimetric method)

To different aliquots of standard solution containing (0.2-3) µg PG , 0.2 ml of 8.4% v/v acetylacetone solution and 1.4 ml of 20% v/v formaldehyde reagents were added in series of 10 ml test tubes . The mixture was heated in a boiling water bath for 50 minutes Then, cooled and diluted to 10 ml with distilled water. The fluorescence intensity was measured at excitation wavelength 392 nm of and emission wavelength of 470 nm using experiment as ablank.

Procedures for capsule

The content of five capsules was emptied out as completely as possible. An accurately weighed amount of the powder equivalent to 100 mg of the drug was was transferred into 100 mL conical flask, extracted with 3 X 30 mL of distilled water and filtered if needed into 100 mL volumetric flask and the solution was completed to 100 mL with distilled water to prepare a stock solution of 100 µg mL^{-1}. This solution was further diluted with the same solvent as appropriate to obtain the

working concentration range. Aliquots covering the working concentration ranges were transferred into a series of 10 mL volumetric flasks and the procedures under the methods were applied. The nominal content of the capsules was determined using the corresponding regression equations or the calibration graphs.

RESULT AND DISCUSSION

pregabalin exhibits a very low UV absorption[19] and as a consequence, poor sensitivity will be achieved by conventional UV spectrophotometric methods. PG contains a primary aliphatic amino group, which is known to react with many color reagents as vanillin, acetylacetone and formaldehyde.

Optimization of the reactions conditions

Method 1 (using Duquenois reagent)

Duquenois reagent is used for the determination of amino group containing compounds[22, 23]. The reagent consists mainly of vanillin, which contains an aldehyde group that could be react with the primary amino group of PG via condensation mechanism to give colored product.

- **Effect of pH and volume of buffer**
  The McIlvain buffer of pH 7.5 was necessary to achieve the reaction and to obtain the color. Trials are carried out to use buffers with different pH values and results obtained showed that the reaction is pH sensitive and showed absorption peaks at pH 6-8 but maximum absorption at pH 7.5 using McIlvain buffer.
  Maximum absorbance intensities were achieved using 2 ml of McIlvain buffer.
- **Effect of Duquenois reagent volume**
  The amount of Duquenois reagent necessary to obtain a linear graph for a drug concentration was studied. It was found that 2ml of Duquenois reagent was sufficient for production of maximum and reproducible color intensity.
- **Effect of Time and heating**
  Time required for complete reaction at room temperature was 40 min. The reaction product was stable for at least 30 min.
  Heating lead to decrease in absorbance so reaction was done at room temperature.
  Study of the reaction time to obtain maximum absorption under the assay conditions is presented in Fig (5)

Method 2 and 3 (Hantzsch reaction)

Hantzsch reaction is a known condensation reaction that was reported in the literatures as a useful pathway for pyrrole and pyridine synthesis[25]. In the same manner, acetylacetone together with formaldehyde react with aliphatic amines by Hantzsch reaction forming a yellow product that can be measured spectrophotometrically or spectrofluorimetrically. The reaction was applied for the determination of certain sulpha- drug[26] , different antibiotics[27], benazepril hydrochloride[28] , tranexamic Acid[29] and benoxinate hydrochloride[30].

The proposed methods for determination of PG (primary amine compound) was based on Hantzsch condensation reaction using acetylacetone as b -diketone and formaldehyde as an aldehyde to form a colored condensation product (Scheme 1). The formed yellow color showed maximum absorption at 333 nm (Fig. 3). Moreover, the reaction product exhibited strong fluorescence at 470 nm (excitation at 392 nm), Fig. 4

Investigation of Assay Parameters

(1) Effect of heating time

Heating at 100°C for7 minutes was sufficient to produce maximum color intensity, while 50 minutes was the optimum heating time using the spectrofluorimetric method and the produced color and fluorescence were stable for 1 hour.

(2) Effect of reagent concentration

0.2 ml of 20% v/v formaldehyde was found to give maximum absorbance using the spectrophotometric method, while 1.4ml produced maximum fluorescence intensity using spectroflourimetric methods.

1 ml of 8.4% v/v acetylacetone was the most suitable concentration, using the spectrophotometric method. While 0.2 ml of the same reagent gave maximum fluorescence intensity for spectroflurometric method .

(3) Effect of pH

Different acetate buffer in a pH range 3.7–6.8 were tried and buffer of pH 5 was the pH of choice for the two methods.

(4) Effect of solvents

Different diluting solvents were used such as water, ethanol, methanol, acetonitrile and acetone but maximum colour intensity was obtained using water as solvent for the reaction.

Stoichiometric Relationship

Job's method of continuous variation usinfg equimolar (2 × 10^{-2}M) solutions of PG , acetylacetone and formaldehyde were used where each two components were used in a complementary volume totaling 2 ml in presence of a constant concentration of the third one and absorbance was measured at the specific ʎmax, Fig. (6).

VALIDATION OF THE PROPOSED METHODS

Linearity

The methods were tested for linearity, accuracy and precision. By using the above colorimetric procedures, linear regression equations were obtained. The regression plots showed a linear dependence of the absorbance over Beer's law range given in Table 1. The table also shows the results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear least-squares treatment of the results, Molar absorptivity ,Sandell's sensitivity. Results of recovery studies with pure drug PG by proposed methods(Table 2) show small values of standard deviation and variance that indicates low scattering of the points around the calibration line and high precision.

Limit of quantitation and limit of detection

The limits of quantitation (LOQ) were determined by establishing the lowest concentration that can bemeasured according to ICH recommendation[31] below which the calibration graph is non linear. The results are shown in Table 1. The limits of detection (LOD) were determined by evaluating the lowest concentration of the analyte that can be readily detected. The results are also summarized in Table 1.

LOQ and LOD were calculated according to the following equations[32]:

LOQ = 10 Sa/b

LOD = 3.3 Sa/b

Where Sa is the standard deviation of the blank, and b is the slope of the regression line.

Precision

The precisions of the assays (intra-day and inter-day) were determined for PG concentrations cited in Table 3. The intra-day precision was assessed by analyzing six replicates of each sample as batch in a single assay run, and the inter-day precision was assessed by analyzing the same sample, as triplicate, in two separate assay runs. The assays, gave satisfactory results (Table 3). This level of precision of the proposed methods was adequate for the quality control analysis of PG.

Accuracy

To test the validity of the proposed methods they were applied to the determination of pure sample of PG over the concentration ranges cited in Table. The results obtained were in good agreement with those obtained using the comparison method[19]. Statistical analysis of the result obtained using student t-test and the variance ratio F-test[32] revealed no significance differences between the proposed and comparison methods regarding the accuracy and precision, respectively (Table 4).

Analytical applications

The results obtained by appling the proposed methods for the determination of PG in its pharmaceutical formulation (pregabalin 75 mg capsules) (Table5) suggest satisfactory recovery. Further, standard addition technique followed to check the validity of the method have given good recoveries of the drug in presence of formulation suggesting a non interference from formulation excepients. Hence, these methods can be recommended for adoption in routine analysis of PG.

CONCLUSION

The proposed methods are simple, inexpensive and sensitive for the determination of PG in bulk as well as in marketed form (capsules). There is no requirement of any sophisticated apparatus as in chromatographic methods. Omission of an extraction step with organic solvents is an added advantage. The methods have been validated in terms of its sensitivity, simplicity, reproducibility, precision and accuracy suggesting its suitablility for the routine analysis of PG in pure form (in bulk analysis) as well as pharmaceutical formulations without interference from excipients.

Table 1: Optical Characteristics and Statistical Data of the Regression

Equations for Determination of pregabalin using the Proposed Methods.
y = ax + b, where x is the concentration of drug in µg/ml; Average of three determination.

Parameters	Method 1	Method2	Method 3
ʎ max/nm	394	333	392(EX.), 470(EM.)
Linearity range (µgml^{-1})	20-180	20-160	0.2-3
Intercept (a)	0.0047	0.0266	363.19
Slope (b)	0.1419	0.0605	169.16
Correlation coefficient (r)	0.9999	0.9998	0.9999
SD	0.576	0.821	0.951
RSD	0.575	0.821	0.947
Molar absorptivity (L mol-1cm-1)	1.165*10^3	10.38*10^3	----------
Sandell's sensitivity (µg/ml/cm2)	0.136	0.015	----------
Limit of detection (LOD) (µgml-1)	6.319	0.545	1.95*10^{-3}
Limitof quantification (LOQ)(µgml-1)	19.148	1.652	5.9*10^{-3}

Table 2: Results of recovery studies with pure drug pregabalin by proposed methods

Parameter	Method 1		Method 2		Method 3	
	Taken µg/ml	Recovery*	Taken µg/ml	Recovery*	Taken µg/m	Recovery*
	20	99.043	2	99.504	0.2	100.187
	40	98.989	4	101.405	0.4	98.857
	80	100.027	6	99.284	1	99.832
	100	100.021	10	99.736	1.5	101.231
	120	100.372	12	99.642	2	100.157
	180	99.539	14	100.992	3	99.675
			16	99.628		
Mean	99.690		100.027		99.990	
SD	0.632		0.821		0.777	
RSD	0.634		0.821		0.777	
SE	0.258		0.311		0.317	
V	0.399		0.675		0.603	

Table 3: Precision of the proposed methods for analysis of pregabalin

Method	Pregabalin (µg ml^{-1})	Intra-day		Inter-day	
		Mean ±S.D	RSD	Mean ±S.D	RSD
Method 1	80	100.352±0.581	0.237	100.350±1.075	0.439
Method2	6	100.333±1.643	0.671	99.500±2.244	0.916
Method 3	0.4	99.492±3.090	1.262	100.208±2.277	0.930

*average of three experiment

Table 4: Determination of pregabalin using proposed methods compared to comparison method

	Proposed method			comparison method[19]
	Method 1	Method 2	Method 3	
Mean ±S.D.	99.69 ± 0.632	100.027 ±0.821	99.990 ±0.777	100.43 ± 1.24
N	6	7	6	5
V	0.399	0.674	0.603	2.188
t	1.236 (2.262)*	0.666(2.228)*	0.604(2.622)*	
F	5.483(6.26)*	3.246(6.16)*	2.386(6.26)*	

*Theoritical values of t and f at P=0.05

Table 5: Application of standard addition technique for the determination of pregabalin in Lyrica® capsules using proposed methods

parameter	Mehod 1 (Duquenois reagent)			Mehod 2 (Spectrophotometric)			Mehod 3 (spectrofluorimetric)		
	Taken µg/ml	Added µg/ml	Recovery*	Taken µg/ml	Added µgml	Recovery*	Taken µg/ml	Added µg/ml	Recovery*
	20	-----	101.170	2	----	100.833	0.2	---	102.891
		20	100.106		2	101.667		0.2	100.763
		40	99.415		4	99.583		0.3	102.093
		60	100.035		6	101.111		0.4	100.216
		100	100.447		8	101.042		0.7	99.4916
		120	100.372		10	99.833		1.2	100.403
					12	100.139			
Mean			100.258			100.601			100.976
SD			0.577			0.762			1.27032
RSD			0.575			0.757			1.25804
SE			0.236			0.288			0.51871
V			0.333			0.580			1.61371

*average of three experiment

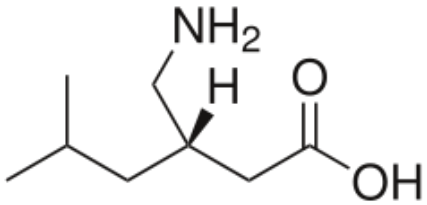


Fig. 1: pregabalin

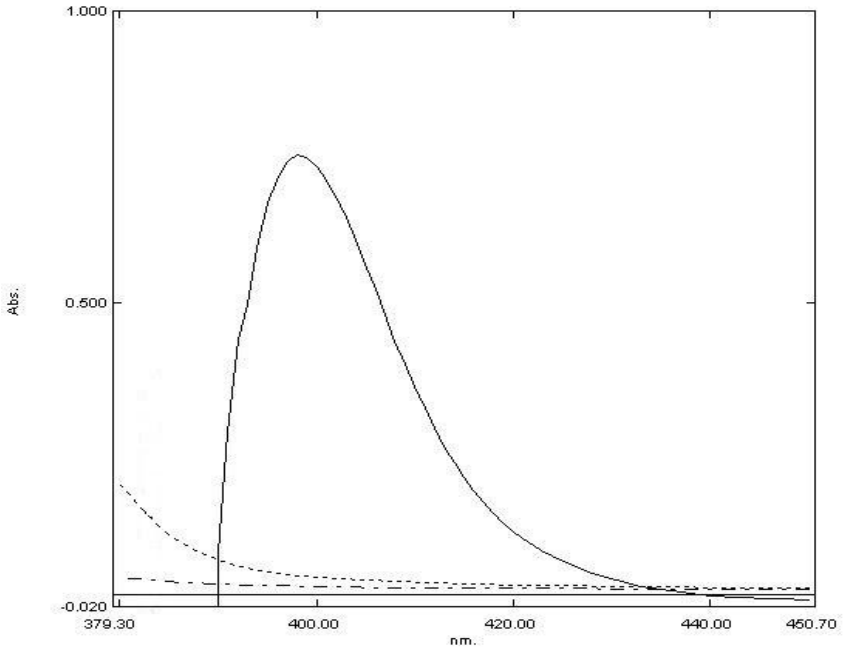


Fig. 2: absorption spectra of reaction product between pegabalin(140 µg ml-1)and Dequenois reagent

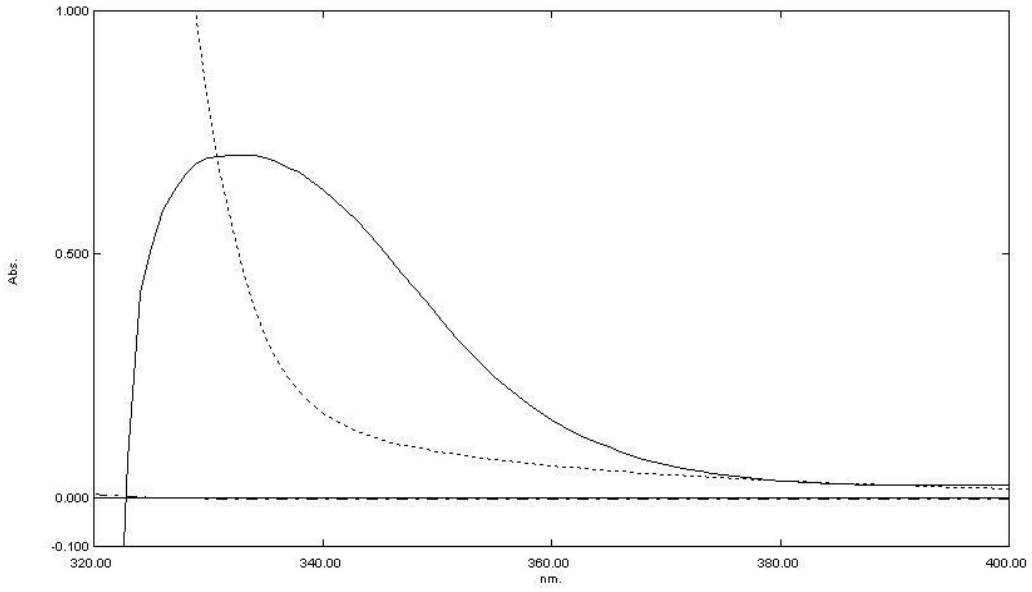


Fig. 3: Absorbance spectra of the reaction between acetylacetone (8.4% v/v), formaldehyde (20% v/v) reagent and 12µg PG.

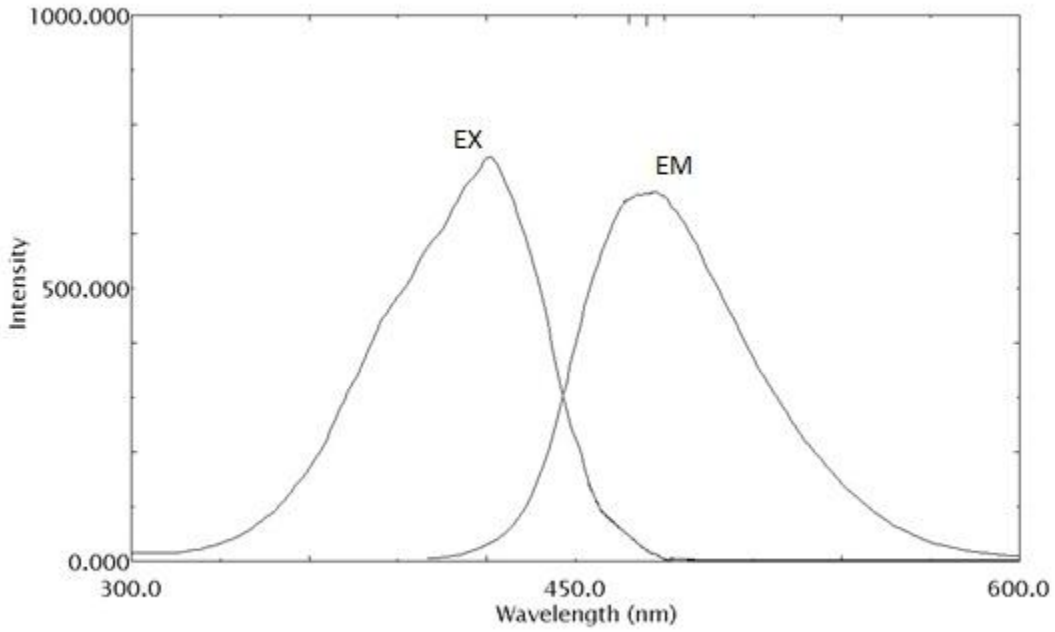


Fig. 4: Excitation (EX) and emission (EM) spectra of the reaction between acetylacetone (8.4% v/v), formaldehyde (20% v/v) reagent and 1.5µg PG

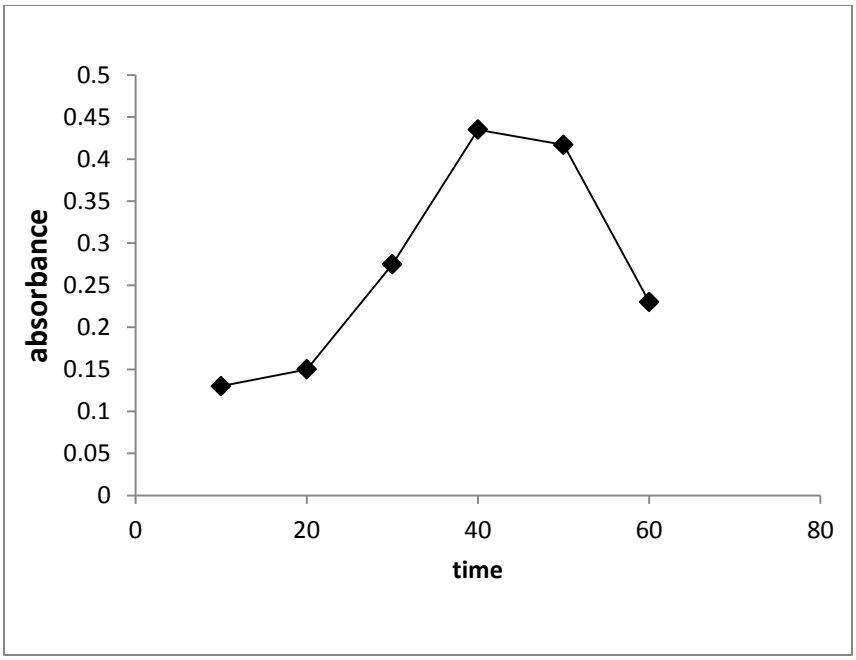
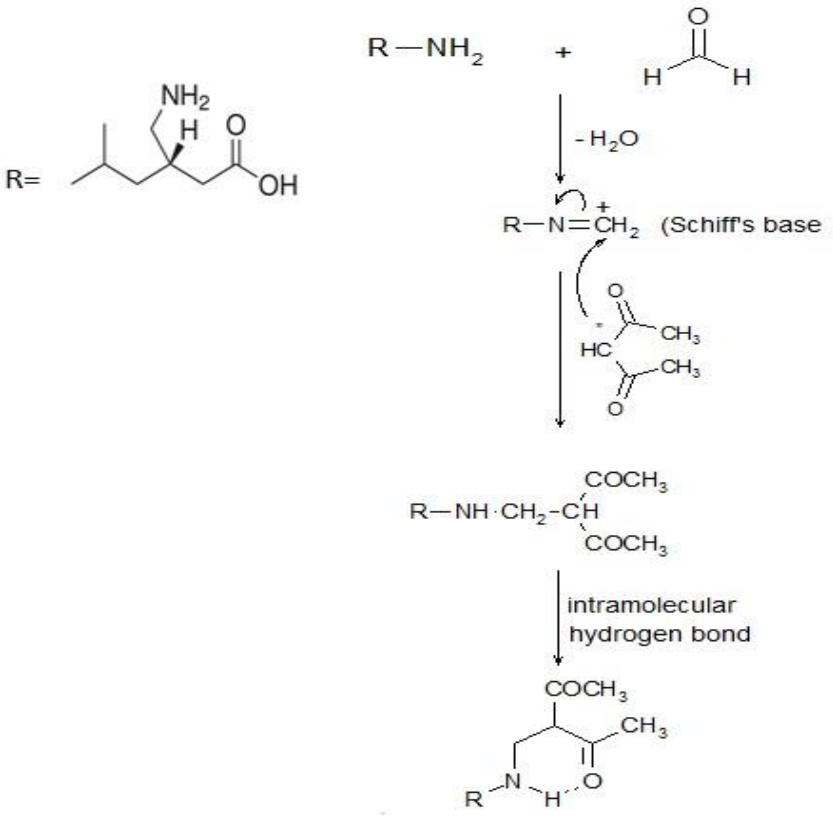


Fig. 5: Effect of time on absorption of 80 µg/ml PG with Duquenois reagent



Scheme 1: Suggested mechanism of the reaction between PG and acetylacetone/formaldehyde

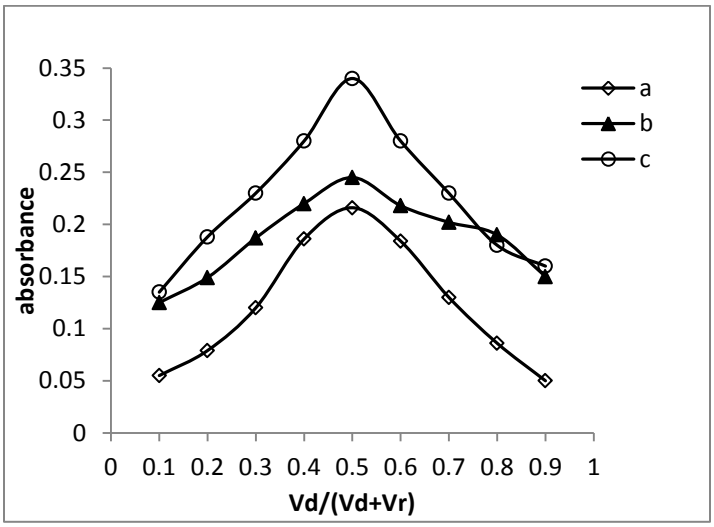


Fig. 6: Continuous variation plot of the reaction between:
(a) 2×10^{-2} M PG and 2×10^{-2} M acetylacetone (in presence of 1 ml formaldehyde).
(b) 2×10^{-2} M PG and 2×10^{-2} M formaldehyde (in presence of 1 ml acetylacetone).
(c) 2×10^{-2} M acetylacetone and 2×10^{-2} M formaldehyde (in presence of 0.2 ml PG)

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