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ORIGINAL ARTICLE

Spectrophotometric method for pregabalin determination: An experimental design approach for method development

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p-Dimethylamino-benzaldehyde;
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Abstract A simple, sensitive and reproducible spectrophotometric method is developed for determining the pregabalin (PGB) content in bulk and in capsule dosage form using an experimental design approach. The proposed method is based on the condensation reaction of PGB (primary amine) with *p*-dimethylaminobenzaldehyde (PDAB) in an acidic medium to form a PGB–PDAB complex. The PGB–PDAB complex shows maximum absorption at 395.80 nm. The proposed method is validated according to the ICH Q2 (R1) guidelines for validation of analytical methods. The percentage purity of PGB in capsule dosage form as determined using the proposed method is 100.05 ± 1.48 whereas the corresponding value by the official method (Indian Pharmacopoeia, 2010) is 100.46 ± 0.41. The *t*-value and *F*-value are calculated for statistical comparison and are found to be 0.60 and 0.08, respectively. The proposed method may recommend for routine quality control analysis of PGB in its pharmaceutical dosage form.

1. Introduction

Pregabalin (PGB), [S-[+]-3-isobutyl GABA or (S)-3-(aminomethyl)-5-methylhexanoic acid, trade name Lyrica] is an anticonvulsant and analgesic medication that is structurally and pharmacologically related to gabapentin (Indian Pharmacopoeia, 2010). PGB is officially approved in Indian Pharmacopoeia (2010) and the use of a liquid chromatographic (LC) method for determining the PGB content in bulk and capsule dosage form has been described. Various LC methods have been reported in the literature (Vermeij and Edelbroek, 2004; Douša et al., 2010; Kannapan et al., 2010; Karavadi and Challa, 2014). A number of UV spectrophotometric methods have also been developed for determining PGB content using different derivatizing reagents. The reagents include potassium iodate and potassium iodide (Gujral et al., 2009), fluorescamine, 2,4-dicloroflurobenzene and 2,3,5,6-tetrachloro-1,

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4-benzoquinone (Shaalan, 2010), ninhydrin (Bali and Gaur, 2011), 1,2-naphthoquinone-4-sulfonate and 2,4-dinitrofluoro benzene (Walash et al., 2011), ascorbic acid and salicylaldehyde (Hammad and Abdallah, 2012) and bromocresol green (Merin et al., 2013). In the current literature Ismail et al. (2016) and Kumble and Narayana (2016) also developed spectrophotometric methods for the determination of bifonazole and febantel, respectively. In methods mentioned in the foregoing, the experimental variables, such as the reagent concentration, reagent volume, heating temperature, heating time and pH of the reaction mixture were optimized using the one variable at a time method (OVAT). As the name suggests, one experimental variable is optimized at a time by varying its concentration or value within a given range, keeping the values of the remaining parameters constant. So, a few parameters are not optimized. Because of this reason the OVAT method gives misleading results and is inefficient for optimizing experimental variables (Lewis et al., 1999). Hence, the use of this method for optimizing experimental variables must be avoided.

Another disadvantage of the OVAT method is that the number of experiments necessary is large, as a result of which the time required and expenses involved are high, with a high consumption of reagents and materials in the experiments.

A systematic and statistical system i.e. an experimental design approach is needed to overcome the limitations of the OVAT method and optimize experimental variables so as to obtain significant and precise analytical results. Therefore, in the present study we used the experimental design approach for screening and optimizing the experimental variables involved in the reaction between PGB and PDAB. PDAB is used to derivate PGB because it lacks chromophore. Previously we had determined the content of cefixime trihydrate using ninhydrin as a derivatizing agent (Wani and Patil, 2013). The method was based on the reaction of the amino group of cefixime with ninhydrin in an alkaline medium to form a yellow-colored derivative (λ_{max} 436 nm). The experimental design approach was utilized to screen and optimize experimental variables involved in the development of UV–Visible spectrophotometric method.

In the current work, we planned to determine the PGB content in bulk and in a pharmaceutical dosage form using PDAB as the derivatizing reagent. An amino compound that lacks chromophore can be assayed spectrophotometrically using a suitable carbonyl reagent. One of the most frequently used reagent PDAB, known as Ehrlich's reagent (Beckett and Stenlake, 1997). Therefore, we selected PDAB as the derivatizing reagent for PGB in the present work. The aim of the present work was to utilize an experimental design approach for developing a UV–visible spectrophotometric method for determining the PGB content using PDAB as the derivatizing reagent. The screening of the experimental variables and their optimization was carried out through a two level fractional factorial design and a response surface methodology, respectively (Minitab Release, 1997).

2. Materials and methods

A PGB sample was obtained from Wockhardt Pharmaceutical Ltd., Aurangabad, India. PDAB and analytical grade concentrated hydrochloric acid (HCl) were purchased from Loba Chemie, Mumbai, India. A commercial pharmaceutical capsule dosage form of PGB (PREGEB 75) was purchased from a local medical shop. A Shimadzu UV–visible spectrophotometer (model 1700) with a pair of matched 1 cm quartz cells and UV-Probe software was used for absorbance measurements.

2.1. Reagents and solutions

2.1.1. PDAB solution (1.5%, w/v)

An accurately weighed quantity of about 150 mg of PDAB was dissolved in 10 mL of methanol. The solution was freshly prepared and protected from light.

2.1.2. PDAB solution (1M)

An accurately weighed quantity of about 1.63 g of PDAB was dissolved in 10 mL of methanol.

2.1.3. Mobile phase

A ternary mobile phase consisting of 0.022 M potassium dihydrogen phosphate (pH 6), methanol and acetonitrile in the proportion of 92:5:3, v/v/v was used for HPLC analysis. Mobile phase was used as diluent for HPLC analysis.

2.1.4. Standard solution of PGB (1000 μg/mL)

An accurately weighed quantity of about 10.0 mg PGB was dissolved in 10 mL of methanol.

2.1.5. Standard solution of PGB (1M)

An accurately weighed quantity of about 1.58 g PGB was dissolved in 10 mL of methanol.

2.1.6. Standard solution of PGB for HPLC analysis (4000 μg/mL)

An accurately weighed quantity of about 40.0 mg of PGB was dissolved in 10 mL of the mobile phase.

2.1.7. Sample solution of PGB for HPLC analysis (4000 μg/mL)

The contents of 20 capsules were removed and weighed. An amount of this powder equivalent to 40.0 mg of PGB was transferred to a 10 mL volumetric flask (flask). The mobile phase was added, and the contents of the flask were sonicated for 10 min. The volume in the flask was made up to the mark with the mobile phase. The solution was filtered through a 0.45 μm filter paper.

2.2. Optimization of reaction variables using experimental design approach

A two level fractional factorial design was used to screen the effect of the PDAB reagent concentration (% w/v) (X_1 (A)), volume of PDAB reagent (ml) (X_2 (B)), volume of concentrated HCl (ml) (X_3 (C)), heating temperature (°C) (X_4 (D)) and heating time (minutes) (X_5 (E)) on absorbance (R) of the PGB–PDAB complex. On the basis of preliminary experiments performed using the conventional method, the following ranges of values were used in the design-X_1: 1–2% w/v; X_2: 1.5–3.5 mL; X_3: 0.05–0.15 mL; X_4: 50–70 °C and X_5: 5–15 min (Table 1). The design matrix of the two-level fractional

Table 1 Experimental factors and response variable for two level full factorial design

Experimental factors	Code	Levels		*P*-value
		Low	High	
PDAB reagent concentration (% w/v)	X_1	1	2	0.084
Volume of PDAB reagent (ml)	X_2	1.5	3.5	0.973
Volume of Conc. HCl (ml)	X_3	0.05	0.15	0.741
Heating temperature (°C)	X_4	50	70	0.695
Heating time (min)	X_5	5	15	0.026
Response	R			
Formation of PGB–PDAB complex (absorbance)		Maximum		

factorial design (1/2 fraction, V resolution, $2^{(5-1)}$ and 16 runs) is shown in Table 2. All the experimental runs were performed in triplicate and the average absorbance is provided in Table 2.

2.2.1. Optimization using response surface methodology

A three-level central composite design (Table 3) was used to optimize X_1 and X_5. The following experimental conditions were maintained constant-X_2: 2.5 mL; X_3: 0.1 mL; X_4: 60 °C and diluting solvent:methanol. The experiment was performed in triplicate and the average absorbance is provided in Table 3. The optimized experimental variables, as determined using the fractional factorial and three level central composite designs were used in the subsequent experiments.

2.2.2. Optimization of reaction variables by conventional method (OVAT)

Different experimental parameters, such as X_1, X_2, X_3, X_4 and X_5, were optimized using the conventional method to obtain the maximum reaction product or absorbance (R). The resulting solutions were scanned in 200–800 nm range. The maximum absorbance of the complex lies in this range, at 395.80 nm.

Table 2 Design matrix of fractional factorial design and response value

Run	X_1	X_2	X_3	X_4	X_5	R (n = 3)
1	2	3.5	0.15	70	15	0.651
2	2	1.5	0.05	70	15	0.630
3	2	1.5	0.05	50	5	0.715
4	1	1.5	0.15	50	5	0.632
5	2	3.5	0.05	50	15	0.571
6	2	1.5	0.15	50	15	0.622
7	1	1.5	0.15	70	15	0.588
8	1	3.5	0.15	50	15	0.561
9	1	1.5	0.05	70	5	0.695
10	2	3.5	0.05	70	5	0.721
11	1	3.5	0.15	70	5	0.531
12	1	1.5	0.05	50	15	0.493
13	2	3.5	0.15	50	5	0.741
14	1	3.5	0.05	50	5	0.681
15	1	3.5	0.05	70	15	0.500
16	2	1.5	0.15	70	5	0.571

2.2.3. Linearity study

Aliquots of the standard solution of PGB (about 0.05–0.6 mL) were transferred into a series of 10 mL flasks. To each flask, 2.5 mL of 1.5% w/v PDAB solution and 0.1 mL of concentrated HCl were added. The solution was mixed thoroughly, heated on a water bath at 60 °C for 10 min. The reaction mixture was cooled in an ice bath and the final volume was made up to the mark with methanol. The solution was mixed, and the absorbance was measured at 395.80 nm against a blank.

2.2.4. Assay procedure

The content of 20 capsules was weighed. An amount of the powder equivalent to 25 mg of PGB was transferred to a 25 mL flask. Methanol was added and the contents of the flask were sonicated for 10 min so as to dissolve them. The volume was made up to the mark in the flask with methanol. The solution was filtered through Whatman filter paper. Aliquot of about 0.1 mL of the solution was transferred to five 10 mL flasks. About 2.5 mL of PDAB solution (1.5% w/v) and 0.1 mL of concentrated HCl was added to each flask. The solution was mixed thoroughly and heated in a water bath at 60 °C for 10 min and cooled in an ice bath. The volume was then made up to the mark with methanol. The solution was mixed and absorbance at 395.80 nm measured against a blank. The PGB content in the pharmaceutical dosage form was calculated using the following formulae:

$$X_{\mathrm{Est}} = \frac{(A_{\mathrm{U}} \times W_{\mathrm{Std}} \times W_{\mathrm{Avg}})}{(A_{\mathrm{S}} \times W_{\mathrm{Sam}})}$$

$$\%\text{Labeled claim} = \frac{X_{\mathrm{Est}}}{X_{\mathrm{LC}}} \times 100$$

where X_{Est} = content of PGB present in powder per capsule; A_{U} = absorbance of the sample solution; A_{S} = absorbance of the standard solution; W_{Std} = weight of the standard (mg); W_{Sam} = weight of the sample (mg); W_{Avg} = average weight of powder in capsule (mg); and X_{LC} = labeled content of PGB per capsule (mg).

2.2.5. Assay procedure of official method

A LC method is recommended in the Indian Pharmacopoeia, 2010 for determining the PGB content in a capsule dosage form. Analysis of the PGB was carried out on a Luna C18 column (250 mm × 4.6 mm, 5 µm) using a ternary mobile phase consisting of 0.022 M potassium dihydrogen phosphate (pH 6), methanol and acetonitrile (92:5:3, v/v/v) at a flow rate of 1 mL/min. The standard and sample solutions were injected for HPLC analysis. Detection of PGB was carried out at 205 nm. The chromatogram was recorded for PGB. This showed a retention time of 13.42 min. Calculations were made from the peak areas of the standard and sample chromatograms.

2.2.6. Accuracy

The accuracy of the proposed method was determined through a recovery study. A known amount of pure PGB was spiked to pre-analyzed capsule formulation. Analysis of PGB was carried out at concentrations of 80%, 100% and 120% and working solution concentration of about 18 µg/mL, 20 µg/mL and 22 µg/mL, respectively. Determination of the PGB content was carried out using the formula mentioned in

Table 3 Design matrix for optimization of method parameters

StdOrder	RunOrder	PtType	Blocks	X_1	X_5	$R(n = 3)$
11	1	0	1	0	0	0.870
13	2	0	1	0	0	0.875
12	3	0	1	0	0	0.891
4	4	1	1	1	1	0.886
10	5	0	1	0	0	0.893
6	6	−1	1	1.414214	0	0.785
7	7	−1	1	0	−1.41421	0.675
1	8	1	1	−1	−1	0.676
9	9	0	1	0	0	0.642
2	10	1	1	1	−1	0.640
8	11	−1	1	0	1.414214	0.588
3	12	1	1	−1	1	0.565
5	13	−1	1	−1.41421	0	0.536

the section 2.2.4 entitled "Assay procedure". The percentage recovery of the proposed method was calculated using the following formula:

$$\%\text{Recovery} = \frac{E}{T+P} \times 100$$

where E = total amount of PGB estimated (mg); T = amount of PGB from pre-analyzed powder of the capsule (mg); and P = amount of pure PGB added (mg).

2.2.7. Precision

The inter-day precision and intra-day precision of the method were determined. A repeatability study (intra-day precision) was performed by analyzing a PGB solution (10 μg/mL) repeatedly within a day. An inter-day precision study was performed by analyzing a PGB solution (10 μg/mL) repeatedly on different days.

2.2.8. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the following formulae:

$$\text{LOD} = (3.3 \times \sigma)/S$$

$$\text{LOQ} = (10 \times \sigma)/S$$

where σ = standard deviation of the response; and S = slope of calibration curve.

2.2.9. Stoichiometry study

A specific volume (about 0.2 mL) of PGB solution (1 M) was transferred to a series of 10 mL flasks. PDAB solution (1 M) was transferred serially (0.2 mL to 3.4 mL in 0.2 mL steps) into the flasks. Concentrated HCl (0.1 mL) was added to each flask. The reaction mixture was thoroughly mixed and heated on a water bath at 60 °C for 10 min. The reaction mixture was cooled in an ice bath and the final volume was made up to the mark with methanol. The absorbance of colored solution was measured at 395.80 nm against the reagent blank.

3. Result and discussion

A spectrophotometric method was optimized for determining PGB content using PDAB as a derivatizing reagent in the present work. The experimental design approach was used to screen and optimize the experimental variables involved in the proposed method. The reaction took place between the carbonyl functional group of PDAB and the free amino group of PGB yields Schiff's base. The reaction proceeds via an attack of the $-NH_2$ group on carbonyl carbon of the O═CH— to form —N═C— with the elimination of a water molecule (Fig. 1) (Beckett and Stenlake, 1997). The PGB–PDAB reaction product showed the maximum absorption at 395.80 nm against the blank (Fig. 1).

3.1. Optimization of reaction variables using experimental design approach

A two level fractional factorial design or Plackett–Burman design (1/2 fraction, V resolution, $2^{(5-1)}$ and 16 runs) was used to evaluate the main effects of five independent factors on the selected response, R. From the Pareto chart of the effects, it was observed that factors X_1 and X_5 had direct effect on the

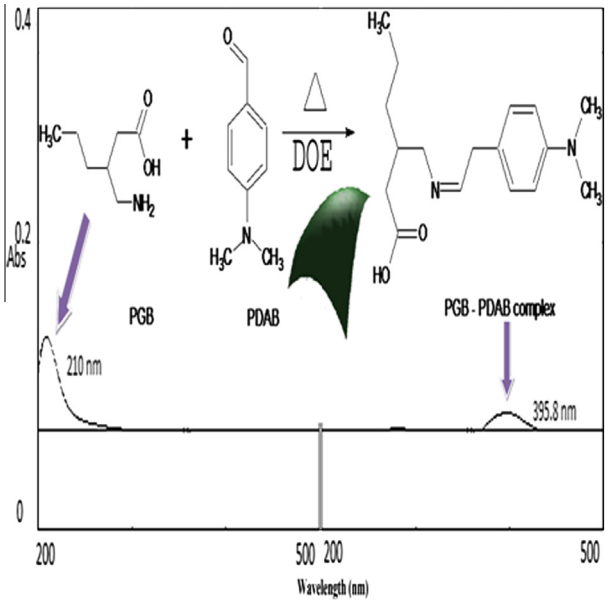

Figure 1 Reaction and UV spectrum of PGB with PDAB.

absorbance, whereas factor X_2, X_3 and X_4 also had direct effects on the absorbance but not significant (Fig. 2). From a normal and half normal plot of the effects and the P value, factors X_1 and X_5 were found to be significant (P value < 0.1) and factor X_2, X_3 and X_4 were insignificant (P value > 0.1) (Table 1).

3.1.1. Optimization using response surface methodology

Using multivariate regression analysis, a fitted full quadratic model was obtained for the average response, R, which is given by the following equation:

$$R = \beta_0 + \beta_1 X_1 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{55} X_5^2 + \beta_{15} X_1 X_5 \quad (1)$$

where R = selected response; β_0 = arithmetic mean response; β_1 and β_5 = regression coefficients of the factors X_1 and X_5, respectively.

A central composite design was used to optimize the reaction variables (X_1 and X_5). From the response surface plot and contour plot (Fig. 3), it was observed that the absorbance increases from the lower left corner to the upper right corner of the plot. In other words, the absorbance increases as the concentration of PDAB and heating time increases simultaneously. This plot suggests that the maximum absorbance was obtained with the concentration range between 1.5 and 2% w/v of the PDAB and a heating time of 10–15 min (Fig. 3) (Table 3). Above a concentration of 1.5% w/v PDAB reagent and more than 10 min heating, the absorbance was constant. Therefore, a 1.5% w/v concentration of PDAB reagent and a heating time of 10 min are the optimized conditions. Tables 4 and 5 show the values of the regression coefficients and their associated P-values. From Tables 4 and 5, it may be observed that the concentration of PDAB affected the response, R, significantly (P value < 0.1).

The plot of the main effects indicates that the PDAB reagent concentration and heating time have similar effects on the response. For both the factors, the response increases as you move from a low level to a medium level, and the response decreases from a medium level to a high level of the factor. However, the interaction plot shows that the increase in the response is greater when the PDAB reagent concentration is in the range between 1.5% w/v and 2.0% w/v than when the PDAB reagent concentration is in the ranges between 0.79% w/v to 0.1% w/v and 2.0% w/v to 2.2% w/v.

The developed model was validated. The experimental results and the predicted values obtained using the polynomial model equation showed that the predicted value matches reasonably with the R-Sq value of 96.67% and R-Sq (adj) of 94.30% of the selected response, R.

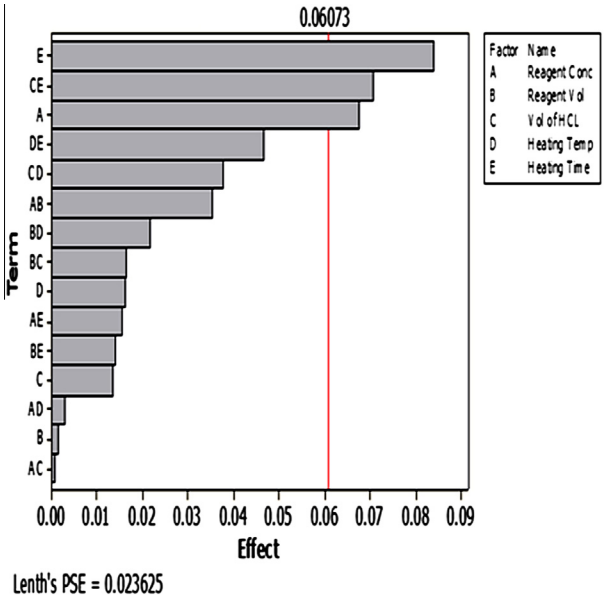

Figure 2 Pareto chart of the effects.

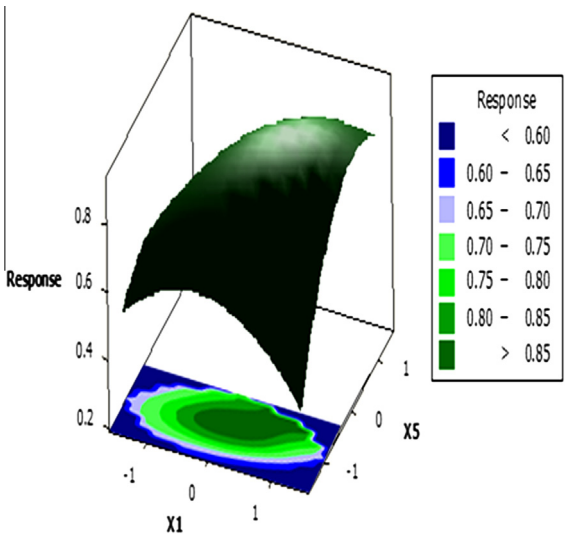

Figure 3 Response surface plot and contour plot of response R.

Table 4 Analysis of variance for response

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.199645	0.199645	0.039929	40.69	0.000
Linear	2	0.046618	0.046618	0.023309	23.75	0.001
X_1	1	0.044698	0.044698	0.044698	45.55	0.000
X_5	1	0.001920	0.001920	0.001920	1.96	0.205
Square	2	0.123956	0.123956	0.061978	63.16	0.000
$X_1 * X_1$	1	0.044423	0.060394	0.060394	61.55	0.000
$X_1 * X_5$	1	0.079534	0.079534	0.079534	81.05	0.000
Interaction	1	0.029070	0.029070	0.029070	29.63	0.001
$X_1 * X_5$	1	0.029070	0.029070	0.029070	29.63	0.001
Residual error	7	0.006869	0.006869	0.000981		
Lack-of-fit	3	0.003789	0.003789	0.001263	1.64	00315
Pure error	4	0.003079	0.003079	0.000770		
Total	12	0.206513				

Table 5 Estimated regression coefficients for response

Term	Coef	SECoef	T	P
Constant	0.87960	0.01401	62.789	0.000
X_1	0.07475	0.01107	6.749	0.000
X_5	0.01549	0.01107	1.399	0.205
$X_1 * X_1$	−0.09318	0.01188	−7.845	0.000
$X_5 * X_5$	−0.10692	0.01188	−9.003	0.000
$X_1 * X_5$	0.08525	0.01566	5.443	0.001

The distribution of the residuals for the response approximately followed the fitted normal distribution, whereas the residuals of the response were randomly scattered in the residual plots. In the present work, the experimental variables were optimized using the conventional method (OVAT) also. The optimum conditions required for the maximum color development were found to be 2.5 mL of 1.5% w/v PDAB solution, 0.1 mL of concentrated HCl and 10 min of heating at 60 °C.

3.1.2. Assay and statistical comparison

The results of the analysis of the marketed formulation using the proposed and official methods are presented in Table 6. The percentage purity of the commercial formulation PREGEB 75® capsule as determined using the proposed method was 100.05 ± 1.48 and that determined using the official method was 100.46 ± 0.41. The two results were compared by calculating the *t*-value and *F*-value, which were found to be 0.60 and 0.08, respectively. From these results, it is concluded that there is no significant difference between the proposed and official methods, indicating that the proposed method is as accurate and precise as the official method.

3.1.3. Stoichiometry study

In this study the weight relationship between PGB, PDAB and the PGB–PDAB complex was determined. The ratio of PGB to PDAB in the complex was determined using the molar ratio method (Miller and Miller, 1993). A graph with a number of moles of PDAB per mole of PGB on the *X*-axis and absorbance on the *Y*-axis shows that a molar ratio of 4:1 (PDAB:PGB) is sufficient for maximum color development (Fig. 4).

Table 6 Optical characteristics, assay result and statistical data of the validation parameters

Parameter	Observation
Medium	Acidic
Color	Yellow
λ_{max} (nm)	395.80
Beer–Lambert's law limit (μg/ml)	5–60
Slope	1.252
Intercept	0.033
Regression coefficient	0.9960
Assay results (n = 5)	
Proposed method	100.05 ± 1.48
Reported method	100.46 ± 0.41
t-value[a]	0.60
F-value[b]	0.08
Accuracy (n = 3)	
Level 80%	101.13 ± 0.15
100%	101.89 ± 0.90
120%	100.01 ± 1.76
Mean	101.03 ± 0.94
Precision (%RSD, n = 5)	
Intraday	0.66
Interday	1.07

[a] *t*0.05%, 4 = 2.776.
[b] *F*0.05%, 4 = 6.39.

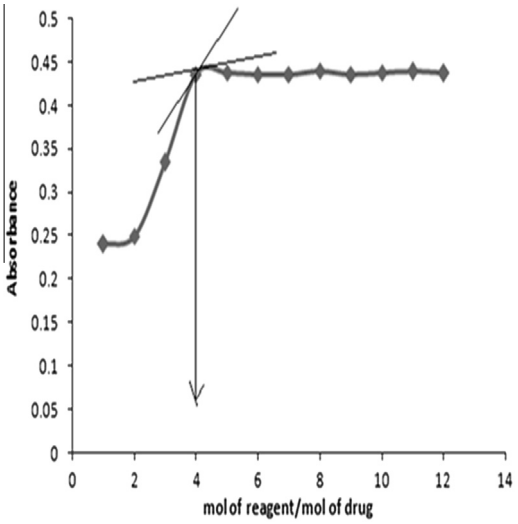

Figure 4 Stoichiometry between PGB and PDAB.

3.2. Method validation

A calibration graph was constructed with PGB concentration on the *X*-axis and absorbance on the *Y*-axis using all the optimized conditions described in the foregoing. A linear relationship was found in the concentration range from 5–60 μg/mL (r^2 = 0.9960) (Table 6). The accuracy study, indicated that the average percentage recovery for PREGEB 75® capsules was 101.03 ± 0.94 (Table 6). The inter-day and intra-day precision values were 1.07 and 0.66 μg/mL, respectively. The % RSD value obtained (< 2) indicates that the proposed method is precise. The LOD and LOQ were found to be 0.025 μg/mL and 0.076 μg/mL, respectively. The solution used in the precision study was stored for 24 h at room temperature. During the storage period, the absorbance of this solution was measured at different time intervals (0 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h). The color intensity of the solution was stable up to 6 h.

4. Conclusion

In the present work optimization of a spectrophotometric method for determining PGB content was optimized using experimental design methodology and the conventional method (OVAT). In the factorial design, out of five factors (X_1, X_2, X_3, X_4 and X_5), two factors (X_1 and X_5) were found to be significant. These significant factors were optimized using response surface methodology. The results of optimization of reaction variables by both the methods were found to be identical ones. However, the experimental design approach was systematic, less time consuming and more cost effective compared with the conventional method. Therefore, the proposed method using an experimental design approach is better for determining the PGB content and can be used for routine quality control analysis of PGB formulations.

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