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Phyto Pigment as Colour Complex-Forming Agent in Novel Colorimetric Method Development for Estimation of Ropivacaine Using Phyto Pigments

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ABSTRACT

Introduction: Anthocyanin group of compounds constitute color pigments having polyphenolic nature which is similar to synthetic dyes like methyl orange, Bromocresol green used in colorimetric methods of quantitative analysis. *Delonix regia* flowers provide a pink color pigment which we thought can be used as a color complex-forming agent.
Objective: In the present work a novel attempt has been made to develop a simple, accurate, and precise colorimetric method using *Delonix regia* flowers pigment for quantitative estimation of ropivacaine
Methodology: Pink color pigment was extracted using the hydroalcoholic mixture. Phytochemical identification, stability study was carried out. Stock and working solutions of ropivacaine were prepared in methanol. The concentration range in final-colored solutions was adjusted in the range of 4 to 20 µg/ml.
Result: The maximum absorbance for the colored complex between *Delonix regia* flowers extract and *ropicine* was measured at 613. Beer's law was followed in the range of 4 to 20 µg/ml. with a correlation coefficient of 0.9994, while the LOD and LOQ were found to be 0.2681 and 0.7962 respectively.
Conclusion: It can be concluded that successful development of simple, accurate, sensitive, and reliable methods has been done for the estimation of ropivacaine.

1. INTRODUCTION

Delonix regia (Fabaceae), an umbrella-shaped tree reaches a height of 40 feet. It is considered one of the most attractive tropical trees in the world. It produces outstanding flame-like scarlet and yellow flowers in spring before the leaves emerge [1]. It is described with the name Gulmohar in Nepal, Pakistan, and India which contains β-sitosterol, tannins, saponins, flavonoids, steroids, alkaloids, carotene, and so on [2,3].

Ropivacaine is a white solid and highly polar compound, chemically (2*S*)-*N*-(2,6-dimethyl phenyl)-1-propylpiperidine-2-carboxamide. It was officially in USP which describes the liquid chromatography method for quantitation. Ropivacaine is chemically(2*S*)-*N*-(2,6-dimethyl phenyl)-1-propylpiperidine-2-carboxamide, used local anaesthetic [8].

Literature survey revealed that work has previously been performed for quantitative estimation of Ropivacaine. Vieira, A.L.N., et al. have developed validated HPLC Method for Quantitative Determination of Ropivacaine in Drug-Delivery Systems. Sridevi *chigurupati et al* have developed an LC Method for the Determination of Ropivacaine Hydrochloride in Bulk Drug and Pharmaceutical Formulations. [8-12].

From the literature, it can be seen that no attempt has been made for colorimetric quantitative estimation of said drugs. In the present work, a novel approach of using a natural color pigment in quantitative estimation API and its formulation has been done. Generally, the pigments obtained from plants are only used only as indicators in carrying out an acid-base titration. In the present work, a successful attempt has been made in the development of a simple, precise, and reproducible colorimetric method for the quantitative estimation of ropivacaine in bulk and injectable dosage forms. [9-12].

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Plant Material

Delonix regia flowers were collected from the local area around the college campus and Shivaji University, Kolhapur during the blooming season from April to July. Further identification and authentication of the plant were done with the help of a herbarium sheet (Specimen No.–SUK2665) from Botany Department, Shivaji University, Kolhapur. Further,

the herbarium sheet was submitted to the Department of Pharmacognosy, Anandi Pharmacy College, Kale, Kolhapur.

2.1.2 Chemical

Ropivacaine was procured from Manus Aktteva Biopharma LLP Ahmedabad, Sodium hydroxide, hydrochloric acid, chlorbutol, methanol and other chemical used were of analytical grade procured from Loba Chemie.

2.2 Method

The fresh flowers were sun-dried first for one day and then in a well-ventilated oven maintained at 40°C overnight. The dried material was packed in plastic bags, sealed under vacuum, and preserved in the laboratory in a deep freezer.

2.2.1 Extraction

The extracts were obtained by soaking dried flowers (0.5 kg) overnight in a hydroalcoholic mixture prepared by using deionized water and methanol (500 mL) acidified with 1ml of citric acid (0.05N) at room temperature (25°C). After 24 h, the extract was filtered and further subjected to the drying process by evaporation on a thermostatic water bath. [12-13].

2.2.2 Spectral analysis of the extract

The hydroalcoholic extract showed dark pink color and was used for spectral characterization.

2.2.3 Chemical tests for anthocyanins

The extracts obtained were screened for anthocyanins by performing different chemical tests.

2.2.4 Color change over different pH ranges

The color changes that might be shown by the pink extract of the D. Regia flower was evaluated at different pH value using buffer solution.

2.2.5 Colorimetric method

A Jasco spectrophotometer (model: UV-630) was used to measure the λmax and absorbance of various extracts of Delonix regia flowers solutions at pH values of 5.0-8.0. Sample (0.06 g each) of various extracts of Delonix regia flowers was dissolved in 10 ml of appropriate

buffer solutions at various pH values. The λmax and absorbance of the solutions were measured in the range of 400–800 nm.

2.2.6 Determination of pKa

The absorbance of Delonix regia flowers extract (0.12%w/v) was determined at the analytical wavelength (AW) in a pH range of 5.0–8.0. As at pH 5.0, the compound exists as a molecular species (unionized form) while at pH 8.0 it occurs in the ionized form. The ionic strength for each buffer used in this study was also calculated. The relationship between the pKa of an indicator (pKin) and pH can be given by Henderson– Hasselbalch equation,

$$pH = pka + [base]/ [acid]$$

The pKa of Delonix regia color was then determined using the following equation,

$$pKa = pH + \log (di-d)/(d-dm)$$

Where, d was observed color solution absorbance at the AW, whereas **di** and **dm** are the absorbances of the ionized and molecular species at the AW.

2.2.7 Stability of Delonix regia color

Reversibility of the color change was also tested for this natural color. For finding the effect of temperature and pH on the stability of pink color Delonix regia extract solutions at two different concentrations (0.1 and 1.0 mg/ml) were prepared in buffer solutions and were studied for 10 days at two different temperatures (23 and 37°C). Samples were kept in screw-capped bottles and shaken continuously (at 80 rpm) in a controlled temperature reciprocating shaker cum water bath over 10 days. The absorbance of the solution at 511 nm was monitored and degradation of Delonix regia flowers color at a particular time was determined.

3. Colorimetric quantitative estimation of Ropivacaine in bulk and tablet formulation

3.1 Selection of drug

Phytochemical investigation of extract of Delonix regia flowers shows the presence of polyphenolic Anthocyanins group of compounds which have nature similar to synthetic dyes like methyl orange, bromocresol Green, etc. It is observed that the synthetic dyes show in general reactivity with amide type of linkage whenever they are used for colorimetric method

development. As Ropivacaine structure contains amide linkage it was thought that it can be used for colorimetric method development.

3.2 Preparation of Standard Stock Solutions

3.2.1 Ropivacaine

A standard stock solution of ropivacaine was prepared by dissolving 50 mg in a few ml of methanol by sonication and the final volume was made up to 100 ml using methanol to get stock solution having a concentration of 500 µg/ml.

3.2.2 Preparation of Standard Working Solutions

Ropivacaine

0.4 ml of stock solution was pipette in a 10 ml graduated volumetric flask. Volume was made up to 10 ml with methanol to get a standard working solution of 20 µg/ml of ropivacaine.

3.2.3 Optimization of reagents and reaction condition

3.2.3.1 For Determination of Ropivacaine

The temperature of the reaction, quantity, concentration, and sequence of addition of reagents were optimized after several experimental trials. Use of extract, solutions having different concentrations like 0.5, 1, 1.5, 2, and 2.5 % were prepared in phosphate buffer pH 7. 1ml of standard working solution of ropivacainehaving concentration 20 µg/ml was pipetted out in different 10 ml graduated volumetric flasks and 1 ml of extract solution having different concentrations was added. The solutions were screened at identified λmax (613 nm). It was observed that 1% solution of extract gives excellent for the formation of the color complex.

The volume of 1% solution to be used was optimized. It was observed that 0.4 ml volume of 1% Extract of Delonix regia flowers solution was found to be optimum for completion of the color complex reaction.

3.3 Procedure for plotting a calibration curve

3.3.1 Ropivacaine

Form stock solution aliquots were pipetted out in different 10 ml volumetric flasks. To each volumetric flask, 0.4 ml of 1% Extract solution of Delonix regia flowers was added and the

volume was then made up to 10 ml with methanol to get final concentrations of 4 to 20 µg /ml. Flasks were kept aside for 5 to 10 minutes for completion of the formation of the reaction complex. The absorbance of the red-colored complex was measured at 613 nm against the reagent blank.

Spectrophotometric analysis

Drug-extract complex for the said drug was scanned in visible light and spectra were determined.

3.4 FTIR study

To find out the occurrence of a complex between Delonix regia extract and drug i.e. Ropivacaine FTIR study was carried out.

3.5 Method validation

3.5.1 Analysis of formulation

An injectable formulation of Ropivacaine taken to prepare a stock solution. The solution was then filtered through Whatman filter paper No.41. Preparation of desired aliquots within the Beer's law limit was prepared and analyzed by the procedure described earlier for the bulk drug. The concentration of ropivacaine present in the sample solution was calculated by using the formula: Abs = A + B* C were, A = Intercept, B = Slope, and C = concentration of the drug.

3.5.2 Recovery Studies

To study the validity and reproducibility of the developed method, recovery studies were performed by adding a known amount of drug (ropivacaine)to the pre-analyzed sample at four different levels and the percentage recoveries were calculated.

3.5.3 Precision

Precision studies were carried out to determine the reproducibility of the developed method. Repeatability was determined by preparing six replicates of three different concentrations of the sample and the absorbance was measured.

3.5.4 Limit of quantification (LOQ) and Limit of detection (LOD)

LOD is the lowest amount of analyte in the sample that can be detected. While LOQ is the lowest amount of analyte in the sample that can be quantitatively determined by suitable precision and accuracy. LOQ and LOD were determined using by using the standard deviation of the response and slope of the related calibration curve as defined in International Conference on Harmonization (ICH) guidelines.

3.5.5 Robustness

Robustness is performed by using MeOH: Water (80: 20). An average of nine determinations was used for robustness.

4. RESULTS AND DISCUSSION

From the present work, it can be observed that the quantitative reaction of ropivacaine with solution of Delonix Regia flowers extract occurs. Anthocyanins have polyphenolic nature; Phenol is mostly is observed to be present in enol form. The formation of color-complex can be attributed to the existence of Keto-enol tautomerism in phenols. As shown by the actual bond energies of the two forms, the tautomeric mixture strongly favors the enols and a very small amount of the keto form in the equilibrium mixture.

The tautomeric form of phenol, in general, is going to be acidic, as electrons on oxygen are going to get delocalized in the aromatic ring, making it easier for removal of H as H^+ i.e. proton making it acidic. While amide due to the presence of lone pair of electrons on nitrogen is going to show basic nature which contributes to the formation of color-complex between amide and the color pigment obtained from Delonix regia.

4.1 Spectral analysis of the extract

The analytical wavelength (λmax) for hydroalcoholic extract was found to be 526 nm respectively. Results are shown in figure 1.

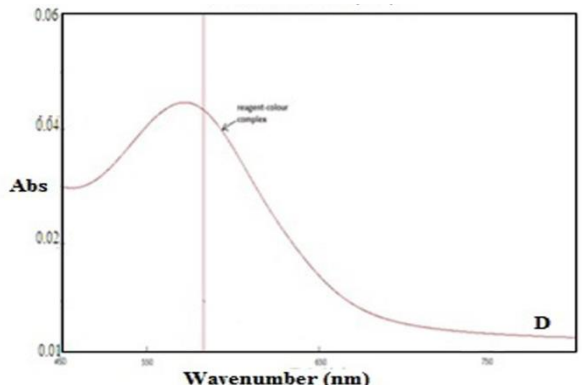

Figure 1 Analytical wavelength (λmax) for hydroalcoholic extract

4.1.1 Chemical tests for anthocyanins

Hydroalcoholic extracts showed the presence of anthocyanins confirmed by the color reactions. Aqueous and methanolic extract showed Blue to violet color with aq. NaOH, yellowish-orange with conc. $H2SO_4$ and red with Mg-HCl are specific tests attributed to the presence of anthocyanins namely Cyanidin3glucoside and Cyanidin3gentiobioside.

4.1.2 Color change over different pH ranges

At a low (acidic) pH it has its original red color but at a basic pH its color changes to pale yellow (Figure 3B). Absorbance and λmax of 0.12% (w/v) solutions of Delonix regia color at different pH values are reported in Table 1 and Figure 3A.

Table 1: Absorbance and λmax of 0.12% (w/v) solutions of Delonix regia color at different pH values*

Sr. No.	pH	λ (nm)	Absorbance
1	3	513.3 ± 0.2	0.15 ± 0.03
2	5	526.6 ± 0.3	0.09 ± 0.05
3	7	509 ± 0.3	0.51 ± 0.10
4	8	558 ± 0.4	0.24 ± 0.08

* Indicates ± SD (n=3)

4.1.3 pKa determination

The mean pKa of extract of Delonix regia flowers dye determined by this spectrophotometric method was calculated to be 7.00 ± 0.16 (mean ± S.D.; n=8). Two different anthocyanins have been isolated from Delonix regia flowers' color. Therefore, the pKa reported here will be considered as a macroscopic pKa for a closely related group present in this natural color. Values of absorbance at different pH, calculated ionic absorbance strength of the buffer (μ), and pKa for an extract of Delonix regia flowers solution are shown in Table 2.

Table 2: Absorbance of Delonix regia flowers solution at 526nm

pH of solution	Calculated ionic absorbance strength of the buffer (μ)	Absorbance (nm)	d (observed at the AW)	Calculated pKa
5	0.0666	1.256	--	--
5.4	0.0702	1.244	1.241	7.09
5.8	0.0753	1.226	1.223	7.16
6	0.0817	1.209	1.207	7.28
6.4	0.0998	0.9266	o.9256	7.57
6.8	0.1288	0.8528	0.8526	7.84
7	0.1446	0.316	0.312	6
7.4	0.1738	0.343	0.342	6.85
7.8	0.1902	0.385	0.381	6.28
8	0.1955	0.4123	--	--

4.1.4 Stability of Delonix regia color

The solution was red at pH 3.0. Dropwise addition of 0.1N NaOH turned the solution pale yellow around pH 8. When 0.1N HCl was added to this pale yellow color slowly, the solution changed back to its original color red at around pH 3.

At pH 3, the extract was found to be stable after 10 days at 23°C and 37°C. However, at pH 7 and pH 8, % degradation of color was found to be increased. This indicates the pH-dependent

degradation of the D. Regia flower extract solution. A higher concentration of extract also increases the chances of degradation Table 3.

Table 3: Effect of temperature and pH on the degradation of aqueous extract of Delonix regia flowers color*

pH	Concentration (mg/ml)	% Degradation of Delonix regia color over 10 days at different temperature	
		23 ± 1.0°C	**37 ± 0.5°C**
3	0.1	4 ± 0.1	6.4 ± 0.5
	1	4.5 ± 0.3	10.3 ± 0.3
7	0.1	10 ± 1.6	19.4 ± 1.2
	1	25 ± 0.4	33.2 ± 1.0
8	0.1	35.5 ± 2.5	54.3 ± 0.4
	1	54.6 ± 0.9	71.1 ± 0.3

* Indicates ± SD (n=3); Degradation (%) = (original concentration−determined concentration) ×100

4.2 Use of Delonix regia color as pH indicator

Chlorobutanol is chlorinated alcohol used as a preservative at a concentration of 0.5% (w/v) in pharmaceutical systems. This compound shows stability in acidic conditions and is hydrolyzed in basic or neutral conditions to form hydrochloric acid with results in a decrease in the pH of the solution. This pH change has also been shown to be dependent on temperature. When exposed to 60 °C, the pH changes in 0.5% (w/v) chlorbutol solution were found to be more than 1 pH unit. Therefore, this system was ideal for testing this pH indicator.

At a low (acidic) pH it has its original red color but at a basic pH its color changes to pale yellow. This color change is reversible with pH at room temperature. The color change in a pharmaceutical solution due to a change in the pH of the solution caused due to an in situ degradation can be visually confirmed even at a low concentration (0.015% w/v) of

chlorbutol solution of Delonix regia used as a pH indicator. Observed spectral scans at 0 hr and after 12 and 24 h are shown in Figure 2C.

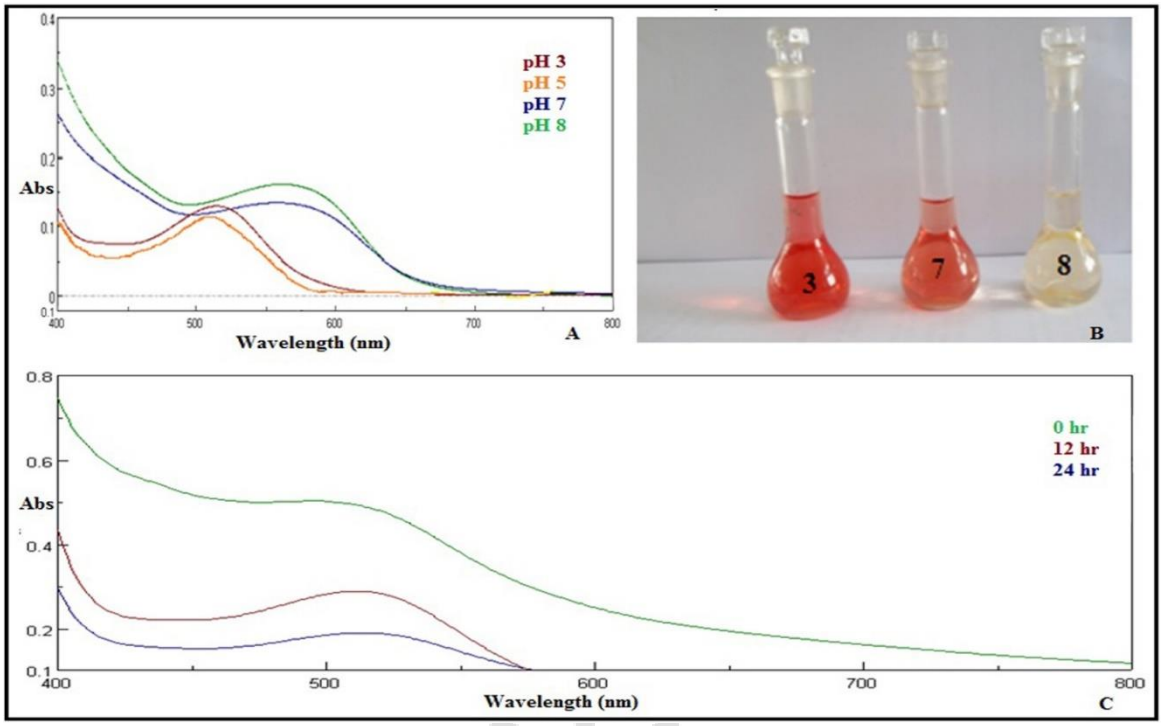

Figure 2 Effect of temperature and pH on the degradation of aqueous extract of Delonix regia flowers color

4.3 Spectrophotometric analysis

UV-Visible spectra of complex formed between D. Regia pigment and ropivacaine are reported in Figure 2C. UV- visible overlain spectra of the Ropivacaine color complex have been shown in Figure 3.

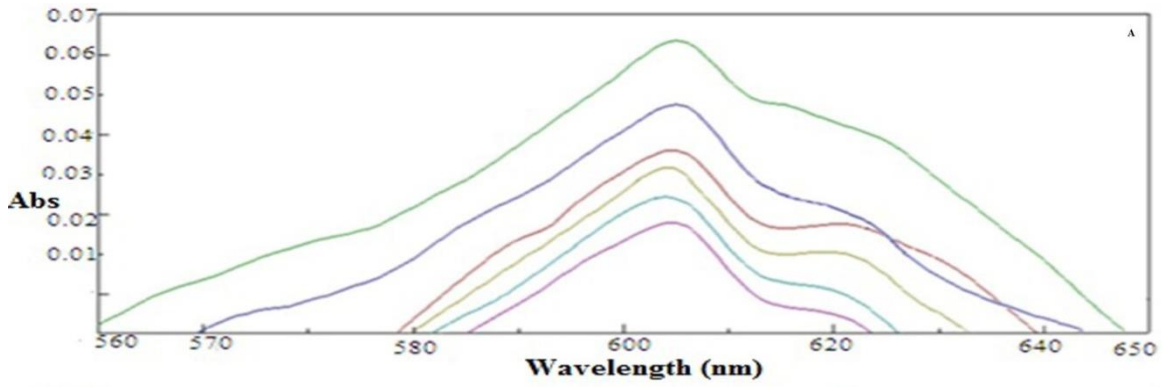

Figure 3 UV- visible overlain spectra for ropivacaine color complex

4.4 FTIR study

FTIR study of pure drugs (Ropivacaine), extract of D. Regia, and complex of extract and drug indicate there are no incompatibilities between extract and drugs. D. Regia extract showed a major peak at 3366.9, 2956.83, 1451.28, and 666.92 cm^{-1} attributed to phenols -OH stretching, aromatic C-H stretching, aromatic -C=C- stretching and aromatic substitution respectively. Ropivacaine-color complex and Ropivacaine-color complex also shows a peak at 3326.28 and 3321.56 cm^{-1} attributed to Phenols, -OH stretching, ultimately supports the no interaction between drug and extract (Figure 4).

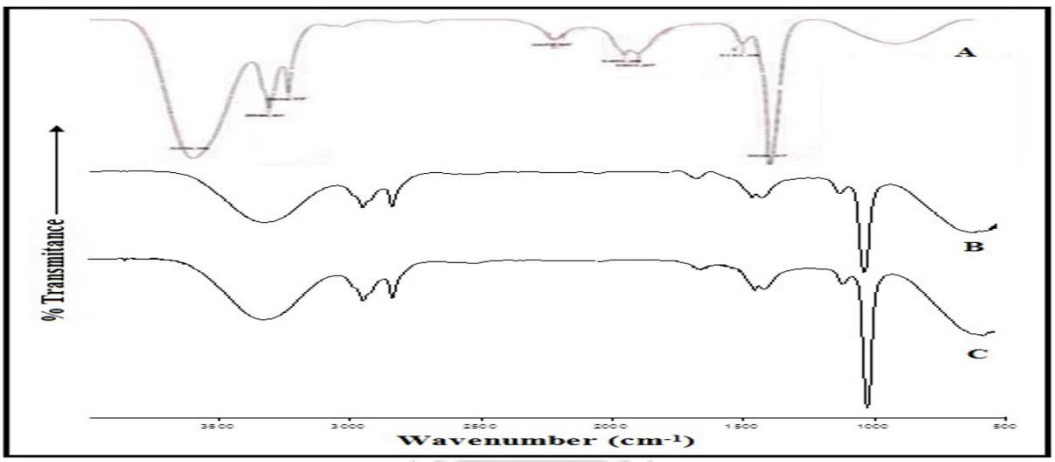

Figure 4 FTIR study of pure drugs Ropivacaine, extract of D. Regia, and complex of extract and drug

4.5 Method validation

4.5.1 Analysis of tablet formulation

The result of the analysis of tablet formulation showed % SD values in the range of 98.34 to 100.58% which indicates the high precision of the method (Table 4).

Table 4: Results of tablet analysis

Analyte	Label claim 5 (0.5%)	% Label claim estimated* (Mean ± SD)	RSD
Ropivacaine	5	98.61 ± 0.03	0.553
Lab sample	5	99.58 ±1.16	--

* Indicates ± SD (n=3)

4.5.2 Linearity study

4.5.2.1 Ropivacaine

A calibration curve was constructed at optimum experimental conditions using absorbance values at 613 nm versus concentration in the range of 4 to 20 µg /ml(Table 5; Figure 6A).

Table 5: Absorbance values for calibration curve of Ropivacaine color complex and Ropivacaine color complex

Sr. No.	Ropivacaine color complex	
	Concentration (µg/ml)	Absorbance
1	4	0.00804
2	8	0.0202
3	12	0.0326
4	16	0.0458
5	18	0.0548
6	20	0.0611

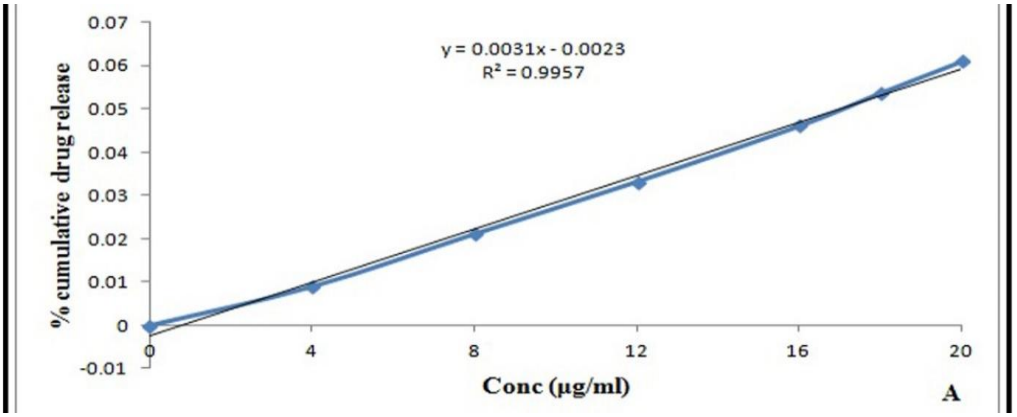

Figure 5 A calibration curve for **Ropivacaine (6A) and Ropivacaine (6B)**

It has shown linear data. The value of the correlation coefficient (r=0.9996) near to 1 indicates good linearity and adherence of the method to Beer's law.

4.6 Recovery studies and Repeatability

The results indicated excellent recoveries for both ranging from 98.65 to 100.43 %. Recoveries obtained for the drug do not differ significantly from 100% showing that there was no interference from common excipients used in the formulation and thus indicates accuracy and reliability of the method. The result of recovery studies and repeatability for the drug is shown in Table 6.

Table 6: Result of recovery study and repeatability*

Analyte	Label claim 5 (0.5%)	% Recovery estimated* (Mean ± SD)	RSD	Repeatability % Label claim estimated* (Mean ± RSD)
Ropivacaine	5	99.22 ± 0.04	0.79	99.34 ± 1.18

*Indicates ± SD (n=3)

4.7 Robustness

The robustness value for ropivacaine was observed to be 99.87 ± 0.35(Table 7). This showed the ability of the method to remain unaffected by the small but deliberate change in reaction conditions.

4.8 LOD and LOQ

LOD and LOQ values are found to be 0.2681 and 0.7962 respectively.

Table 7: Results of robustness, LOD, and LOQ

Parameters	**Ropivacaine**
Robustness	
Label claim 5 (0.5%)	5
% Label claim estimated * (Mean ± % SD)	99.87 ± 0.35
RSD	0.21
LOD (µg/ml)	0.2681
LOQ (µg/ml)	0.7962

* Indicates ± (n=9); RSD - Relative Standard Deviation

5. CONCLUSION

From statistical results of the developed method, it can be concluded that an accurate, precise, reproducible colorimetric method for quantitative estimation of ropivacaine in bulk and dosage form has been developed using Delonix regia flowers extract as a color complex-forming agent.

Conflict of interest

The authors declare that they have no conflict of interest.

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