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Development and Validation of UV Spectrophotometric Method for Simultaneous Estimation of Pregabalin and Etoricoxib in Bulk and Pharmaceutical Dosage Form and the Extension of the Developed Colorimetric Method for Estimation of Pregabalin using Smartphone Application

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ABSTRACT

A simple, sensitive, accurate, precise and reproducible method has been developed for simultaneous estimation of Pregabalin and Etoricoxib in bulk and pharmaceutical dosage form (bilayer tablet). In the method, the methanol and phosphate buffer (pH 7.2) were used as solvent as the drugs could not be extracted from bilayer tablet with single organic solvent. Also, pregabalin is not having chromophore, so it does not show absorbance in UV range. So here the pregabalin was derivatized using ethanolic ninhydrin which shows absorption maximum at 576 nm. Etoricoxib shows the absorption maximum at 284 nm. Beer Lambert's law was obeyed over a concentration range of 2-10 µg/ml for Pregabalin (r^2 = 0.9991) & 4-24 µg/ml for Etoricoxib (r^2 = 0.9998). The developed method was successfully applied for the estimation of Pregabalin and Etoricoxib in commercial product bilayer tablet. The assay was found to be 99.77 and 99.96 % for Pregabalin and Etoricoxib respectively. The developed method has been validated with respect to linearity, range, accuracy & precision. Also, the developed colorimetric method was extended for the estimation of Pregabalin using mobile phone/ smartphone application and was successfully used for the assay of pharmaceutical dosage form.

Keywords: Pregabalin, Etoricoxib, Ninhydrin, UV spectrophotometric, Bilayer tablet, PhotoMetrix

INTRODUCTION

Pregabalin is chemically (3S)-3-(amino methyl)-5-methylhexanoic acid. [1] It is structurally similar to gamma-aminobutyric acid (GABA) - an inhibitory neurotransmitter. [2] The structure of Pregabalin is shown in the Figure 1. [3] Pregabalin is indicated for the management of neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia, fibromyalgia, neuropathic pain associated with spinal cord injury, and as adjunctive therapy for the treatment of partial-onset seizures in patients 1 month of age and older. [2]

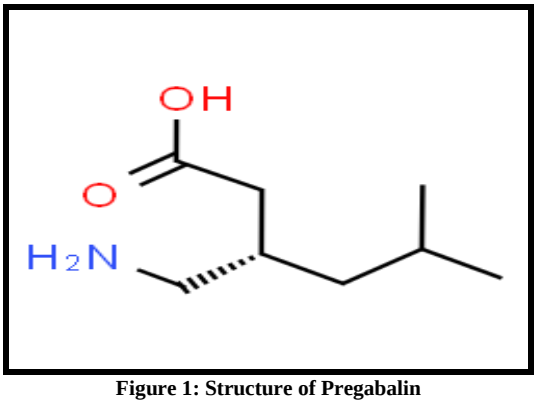


Figure 1: Structure of Pregabalin

Etoricoxib is chemically 5-chloro-2-(6-methylpyridin-3-yl)-3-(4-methylsulfonylphenyl) pyridine. [4] It is a synthetic, nonsteroidal anti-inflammatory drug (NSAID) and COX 2 selective inhibitor. [5] The structure of etoricoxib is shown in the figure 2. [6] Current therapeutic indications of Etoricoxib are treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, chronic low back pain, acute pain and gout. [5]

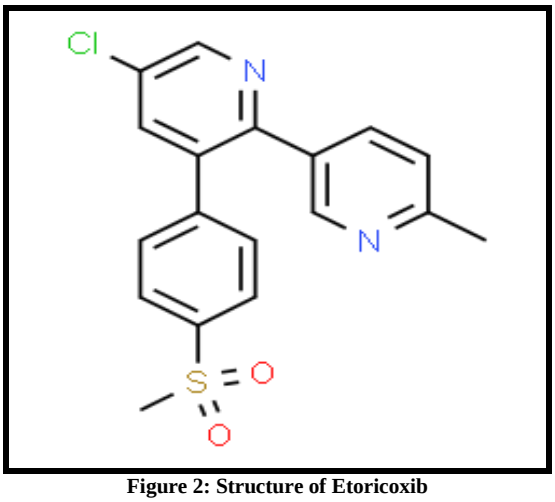


Figure 2: Structure of Etoricoxib

In November,2019, the CDSCO has approved the combination of these two drugs as a bilayer tablet for the treatment of chronic low back pain associated with neuropathic component. [7] Various analytical methods are reported for the estimation of individual drug as well as in combination with other drugs. But there is only one UV- Spectrophotometric method reported for estimation of this combination which is without derivatization of Pregabalin and is based on standard addition method. [8]

In this study, a simple, precise, accurate and sensitive UV-spectrophotometric method was developed for simultaneous estimation of Pregabalin and Etoricoxib which is without addition of standard drug. There were two major challenges in the development of the method. The first is that, as Pregabalin is having no chromophoric group, it doesn't show absorbance in the UV range, therefore it requires the derivatization. The second is the extraction of drugs from the pharmaceutical formulation. As the formulation is bilayer tablet, where the pregabalin is prolonged release, the drug is trapped in polymer. So could not be extracted with single organic solvent. These challenges were overcome by derivatizing the pregabalin with Ninhydrin and by extracting the drug in the mixture of organic solvent and phosphate buffer.

Also, the developed colorimetric method of Pregabalin was extended for the estimation of drug by the mobile phone/smart phone application. Smartphone-based colorimetry has been gaining relevance because of the widespread advancement of devices with increasing computational power, their relatively low cost and portable designs with user-friendly interfaces, and their compatibility with data acquisition. [9] Various methods has been reported for the estimation of drugs by using smart phone application in which mostly the RGB (Red, Blue and Green) principle has been used. [10-13] In this study, the mobile phone application, called PhotoMetrix, which employs the techniques of simple linear correlation for univariate analysis and principal components analysis (PCA) for multivariate exploratory analysis was used. [14] This PhotoMetrix application is available free in Google Play Store. [14] The method was based on the detection of colour intensities and the evaluation of relationship between measured colour and concentration of sample. [10]

MATERIALS & METHODS

Apparatus and Software:

Shimadzu UV-1700 double beam spectrophotometer connected to a computer with Shimadzu UV-Probe 2.10 software installed was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-800 nm. The samples were weighed on an electronic balance (A×120) by Shimadzu. Smart phone

having application PhotoMetrix was used to take images.

Chemicals and Reagents:

Pregabalin and Etoricoxib (API) were obtained from Zydus Cadila Healthcare Limited as gift samples. Nucoxia PG bilayer tablet was purchased from local medical store.

Preparation of Ethanolic Ninhydrin solution (3% W/V): 3 gm of Ninhydrin was dissolved in 95 volume of ethanol and 5 volumes of Glacial acetic acid.

Preparation of phosphate buffer (pH 7.2): 50 ml of 0.2 M potassium dihydrogen phosphate was placed in 200 ml volumetric flask. To that 34.7 ml of 0.2 M NaOH was added and volume was made up to the mark with water.

Methanol was used as diluent.

(All chemicals used in the present study were of analytical grade.)

Preparation of standard stock solution:

5 mg of Pregabalin and Etoricoxib was weighed accurately and transferred into separate 50 ml volumetric flask. To each flask 10 ml of methanol was added and shaken well. Then 20 ml of phosphate buffer (pH 7.2) was added and sonicated for 15 min. Then volume was made up to 50 ml with phosphate buffer. This will give the conc. of 100 µg/ml of Pregabalin and Etoricoxib.

Preparation of sample solution:

20 tablets of formulation (Nucoxia PG) were accurately weighed and powdered. An amount of powder equivalent to 5 mg of Etoricoxib was weighed and transferred into 50 ml volumetric flask. 10 ml of methanol was added to that and shaken well. Then 20 ml of phosphate buffer (pH 7.2) was added and sonicated for 15 minutes. Then solution was made up to the mark with buffer and filtered. This will contain 100 µg/ml of Etoricoxib and 125 µg/ml of Pregabalin.

Selection of wavelength for Etoricoxib:

1 ml of the stock solution of Etoricoxib was transferred into 10 ml of volumetric flask and was made up to the mark with methanol. The resulting solution was then scanned between 200-400 nm. The wavelength corresponding to maximum absorbance was found to be 284 nm.

Selection of wavelength for Pregabalin:

Pregabalin doesn't show absorbance in UV region, even at 100 µg/ml concentration. Therefore, it is derivatized using Ninhydrin. For preliminary test, to 1 ml of the stock solution, 1.5 ml of 1% ethanolic ninhydrin solution was added and heated for 5 min at 60°C. The solution turns purple which was scanned between 400-800 nm. The wavelength corresponding to maximum absorbance was found to be 576 nm.

Optimization of the colorimetric method:

To achieve the optimum conditions for this method, the following parameters were studied by keeping other parameters constant.

Optimization of Concentration of Ninhydrin solution:

To 1 ml stock solution of Pregabalin, 1ml of different conc. (1-5 %) of ninhydrin was added. The reaction mixtures were heated for 10 min on a water bath at 60°C. The coloured product was diluted up to 10 ml with methanol and the absorbance was measured against a reagent blank at 576 nm. The results showed that the highest absorbance was obtained with 3 % of ninhydrin solution (Figure 3).

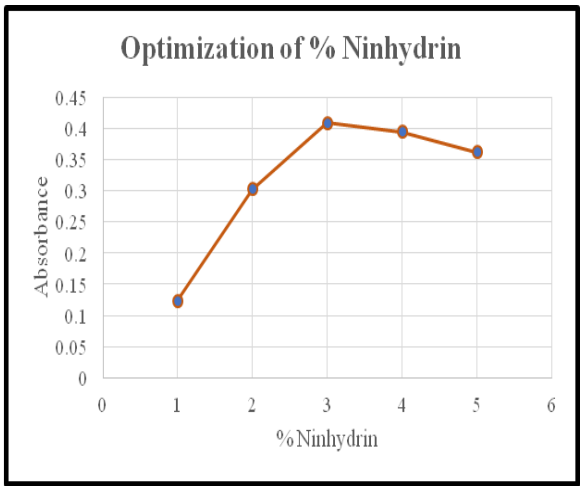


Figure 3: Optimization of % Ninhydrin

Optimization of Ninhydrin Volume:

To 1 ml stock solution of Pregabalin, different volumes (0.2-2.5 ml) of 3 % Ninhydrin were added. The reaction mixtures were heated for 10 min on a water bath at 60°C. The coloured product was diluted up to 10 ml with methanol and the absorbance was measured against a reagent blank at 576 nm. The results showed that the highest absorbance was obtained with 2 ml of ninhydrin solution (Figure 4).

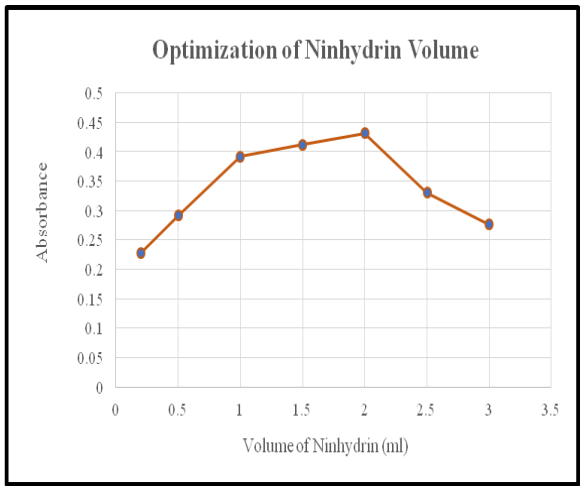


Figure 4: Optimization of Ninhydrin Volume

Optimization of Temperature:

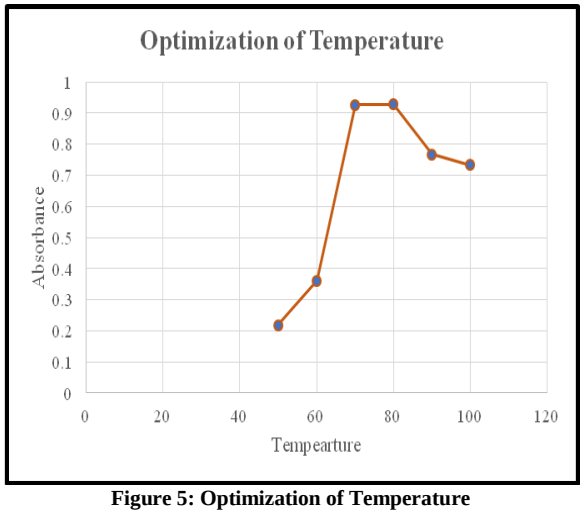


Figure 5: Optimization of Temperature

To 1 ml stock solution of Pregabalin, 2 ml of ninhydrin solution (3% w/v) was added. The reaction mixtures were heated for 10 min at 50-100°C. The coloured product was diluted up to 10 ml with methanol and the absorbance was measured against a reagent blank at 576 nm. The results showed that the highest absorbance was obtained at 70±2°C (Figure 5). The developed colour was stable for 48 h.

Optimization of Heating time:

To 1 ml stock solution of Pregabalin, 2 ml of 3 % w/v of ninhydrin solution was added. The reaction mixture was heated on a water bath at 70°C for 5-45 min. The coloured product was diluted up to 10 ml with methanol and the absorbance was measured against a reagent blank at 576 nm. The results showed that the highest absorbance was obtained with 25 min (Figure 6).

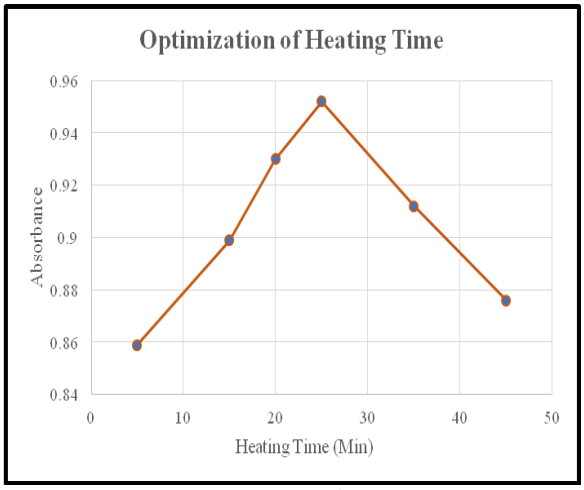


Figure 6: Optimization of Heating Time

The table 1 describes the optimized value of all 4 parameters.

Table 1: Optimized conditions for the colorimetric estimation of Pregabalin

Parameter	Optimized value
% Ninhydrin	3%
Volume of Ninhydrin	2 ml
Temperature	70℃
Heating time	25 min

Reaction Mechanism:[15]

Reaction of ninhydrin with amines, alpha amino acids, peptides, and proteins yields an aldehyde with one carbon atom less than the alpha-amino acid; and carbon dioxide in stoichiometric amounts and varying amounts of ammonia, hydrindantin and a chromophoric compound known as Ruhemann's Purple (2-(3-hydroxy-1-oxo-1H-inden-2-ylimino)-2Hindene-1,3-dione). This pigment serves as the basis of detection and quantitative estimation of alpha-amino acids. Mechanism proposed (Figure 7) for

the reaction involves removal of a water molecule from ninhydrin hydrate 1 to generate 1,2,3-indantrione 2 in the first step, which then, forms a Schiff's base with the amino group of pregabalin resulting in the ketamine 3. Removal of the aldehyde RCHO generates an intermediate amine 4 (2-aminol,3-indandione). Condensation of this intermediate amine with another molecule of ninhydrin follows to form the expected chromophore 5 (Ruhemann's Purple).

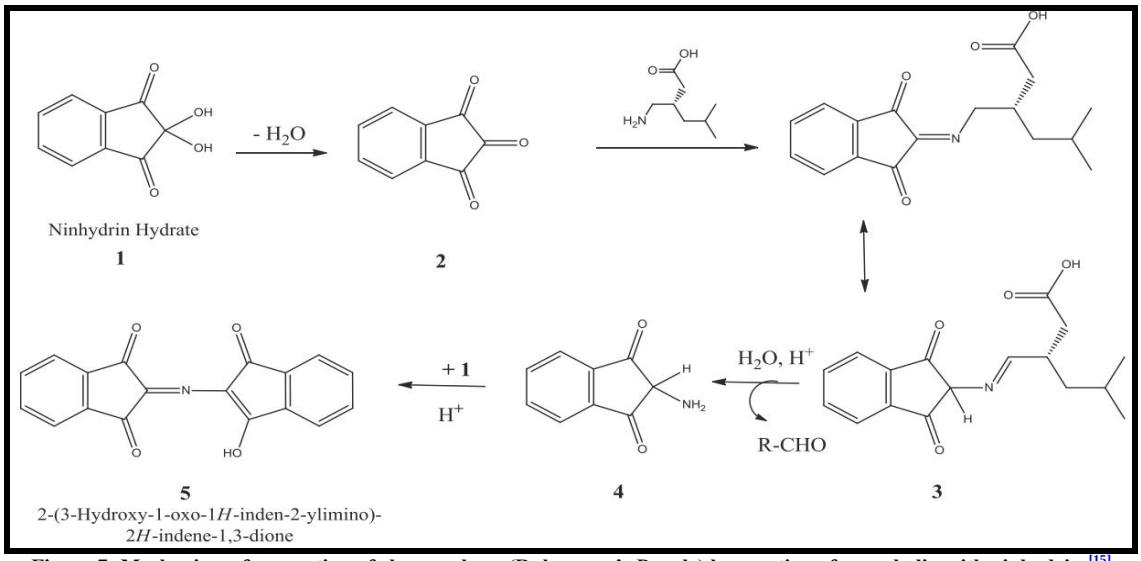


Figure 7: Mechanism of generation of chromophore (Ruhemann's Purple) by reaction of pregabalin with ninhydrin.[15]

Preparation of calibration graph:

Pregabalin:

Aliquots of standard solution of Pregabalin corresponding to 2-10 µg/ml was taken into 10 ml volumetric flask. To each flask 2 ml of 3% Ninhydrin solution was added and solution was heated for 25 min at 70 ℃. The solution was allowed to cool at room temperature and then volume was made up to 10 ml with methanol. The absorbance of the solution was measured at 576 nm against blank.

Etoricoxib:

Aliquots of standard solution of Etoricoxib corresponding to 4-24 µg/ml was taken into 10 ml volumetric flask and was made up to the mark with methanol. The absorbance of each solution was measured at 284 nm.

The calibration curve was plotted at their corresponding wavelengths.

Analysis of marketed formulation:

From the prepared sample solution of formulation, 0.6 ml was transferred into 10 ml volumetric flask and was made up to the mark with methanol. The absorbance of solution was determined at 284 nm for the estimation of Etoricoxib. From the remaining sample solution again 0.6 ml was transferred into 10 ml volumetric flask and treated in the same manner as given for working standard of Pregabalin and absorbance was measured at 576 nm. The absorbance of the drugs was calculated by standard curve method.

ESTIMATION OF PREGABALIN USING SMARTPHONE APPLICATION:

Experimental Setup:

A self-designed box was built in the lab to improve accuracy and precision of the measurements. On the upper side of the box, an LED bulb was fitted to provide consistent incident light source. All the inner side wall of the box were covered with white paper to provide full reflection of the light. The front side is made in a manner that it can be open to insert a cuvette inside the box (Figure 9A). In the front side of the box, a small square shaped hole was made exactly in the middle to allow the camera to take photo of the object placed inside the

box. Also, A cuvette holder was made from thermocol and was fixed in the middle of the box. The entire experimental setup is illustrated in the figure 8.

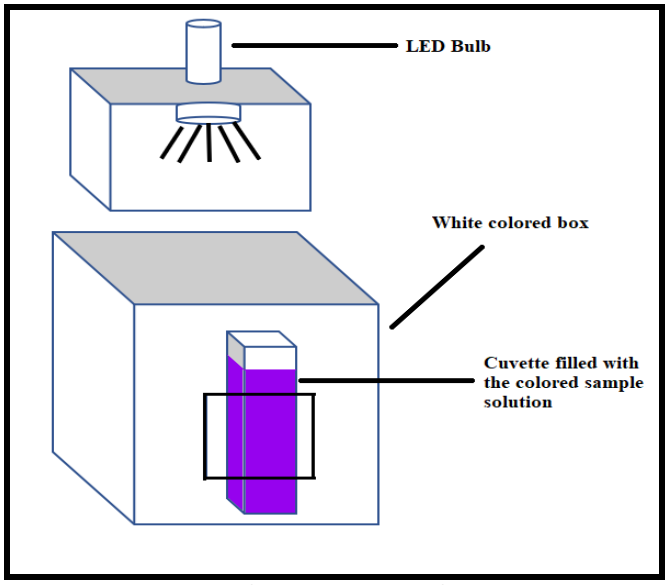
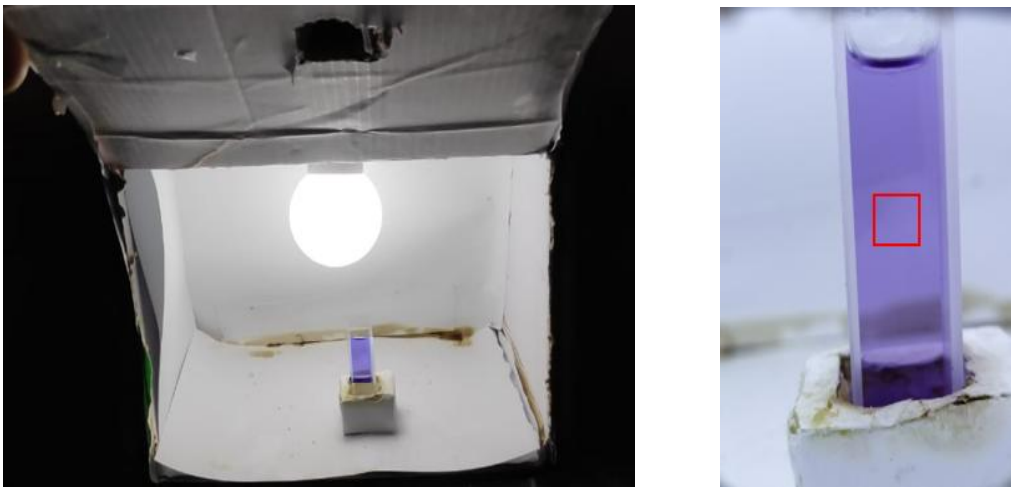


Figure 8: Illustration of experimental setup for image acquisition



A **B**

Figure 9: A. Cuvette placed in the arranged setup
B. Image captured by mobile phone camera in the arranged setup

Preparation of calibration graph by smart phone application:

Aliquots of standard solution of pregabalin corresponding to 2-10 µg/ml was taken into 10 ml volumetric flask. To each flask 2 ml of 3% Ninhydrin solution was added and solution was heated for 25 min at 70̊ C. The solution was allowed to cool at room temperature and then volume was made up to 10 ml with methanol.

Once the standard solutions were prepared, the images were captured one by one in the PhotoMetrix Pro application. The interface of the application as well as the options to be choose in stepwise manner was shown in the figure 10. In application first Univariate Analysis, then in Univariate Analysis Vector RGB was selected. Then once you click on calibration, the app will ask about the number of samples. Here, in number of samples 6 was written (1 blank and 5 standards). Then first the blank solution was filled in the cuvette and was inserted in the box and after writing 0 in the concentration section, image was captured by putting the camera at the middle hole of the box. In same manner one by one the image was captured of all standard solution

in an increasing order of concentration. Then the save button was clicked and the calibration graph as well as regression equation was shown by the application itself.

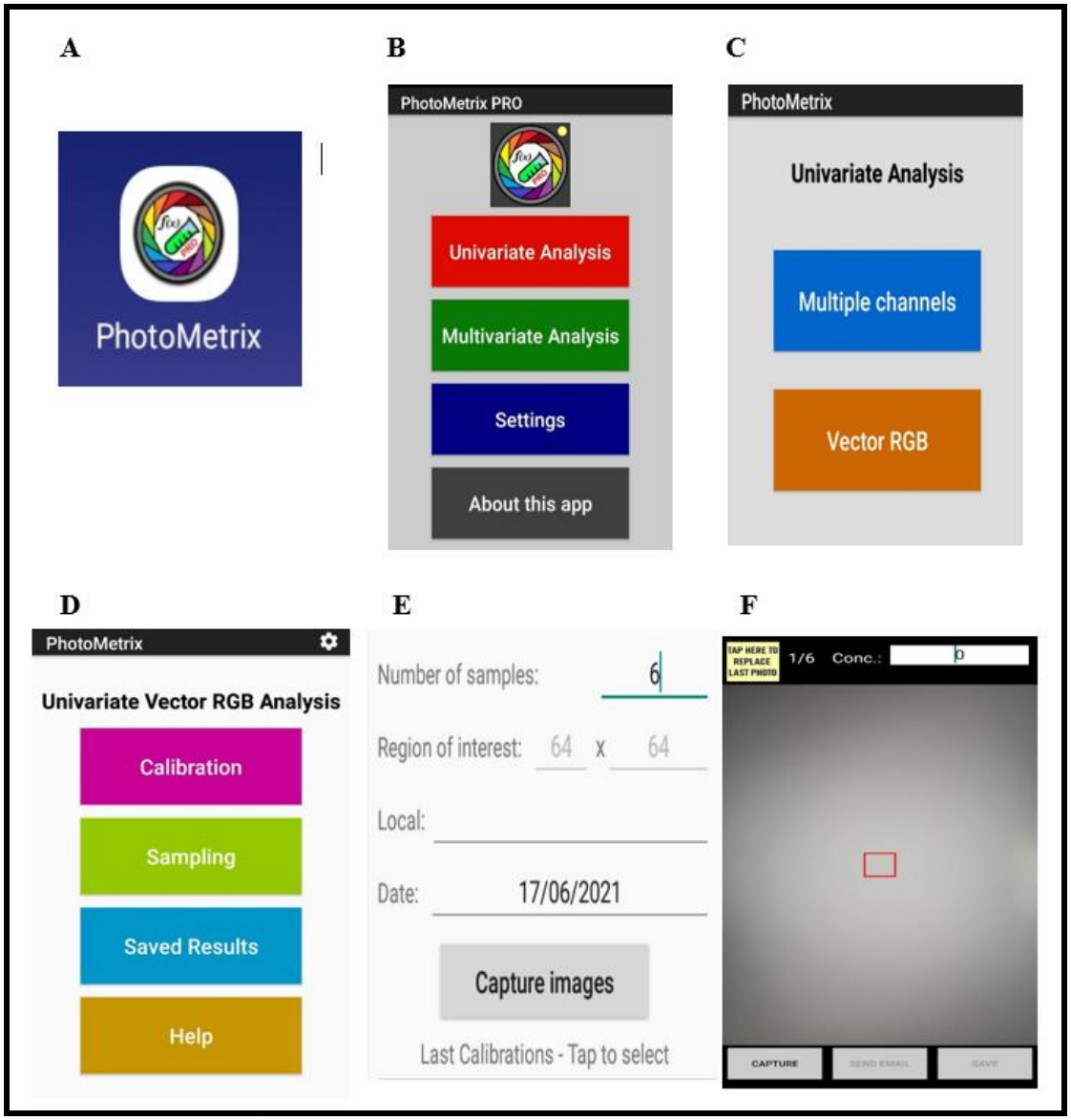


Figure 10: Graphic interface of the PhotoMetrix Application and steps to generate calibration graph in application

Once the regression equation was obtained, the concentration of the sample solution from formulation was estimated. Here, instead of calibration, sampling button was clicked and the image of sample solution prepared for assay was captured in the manner similar to standard solution. Then save button was clicked and the concentration of sample was given by application from the generated calibration graph.

RESULT AND DISCUSSION

Method Validation:[16]

1. Linearity:

Pregabalin was linear with the concentration range of 2-10 µg/ml at 576 nm and Etoricoxib showed the linearity in the range of 4-24 µg/ml at 284 nm, by obeying Beer's law (Figure 10 and 11). A calibration curve was plotted between concentration Vs absorbance. The plot was found to be linear and shown in the figure 13 and 14. Also, the statistical data are shown in the table 2.

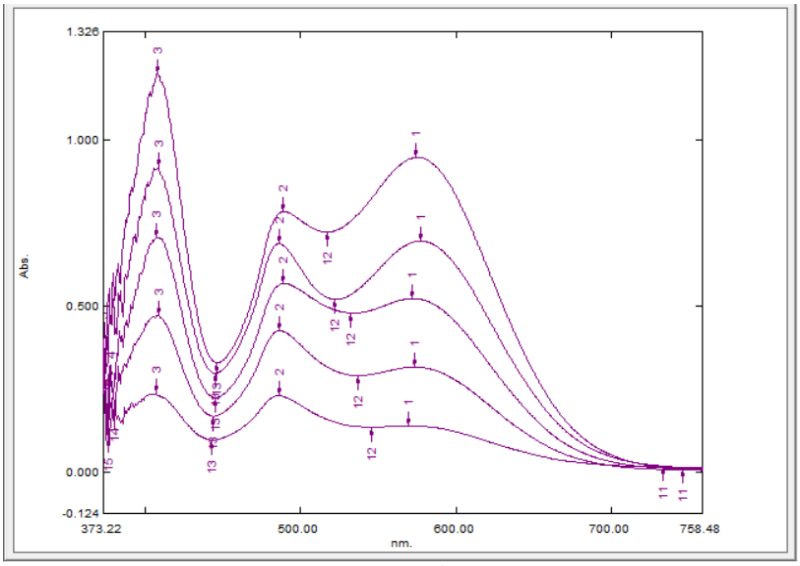


Figure 11: Overlay UV Spectra of Pregabalin (2-10 µg/ml)

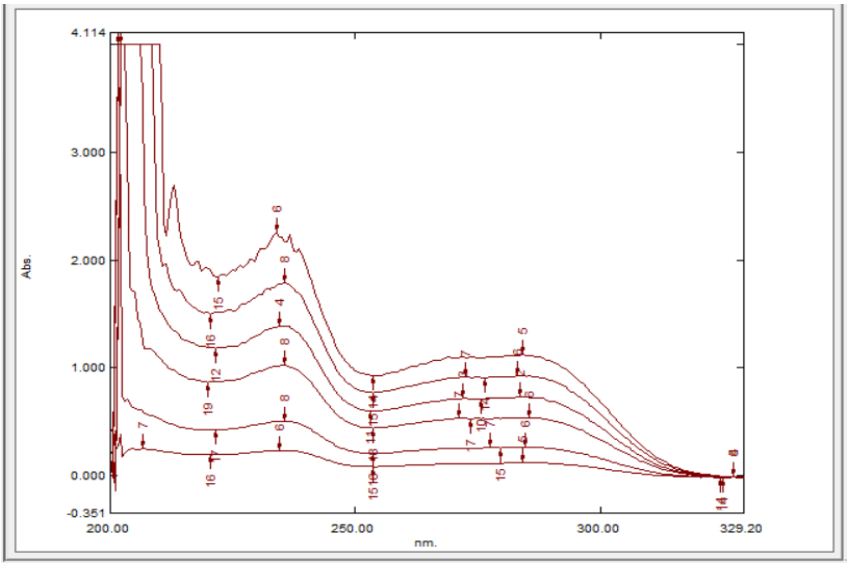


Figure 12: Overlay UV Spectra of Etoricoxib (4-24 µg/ml)

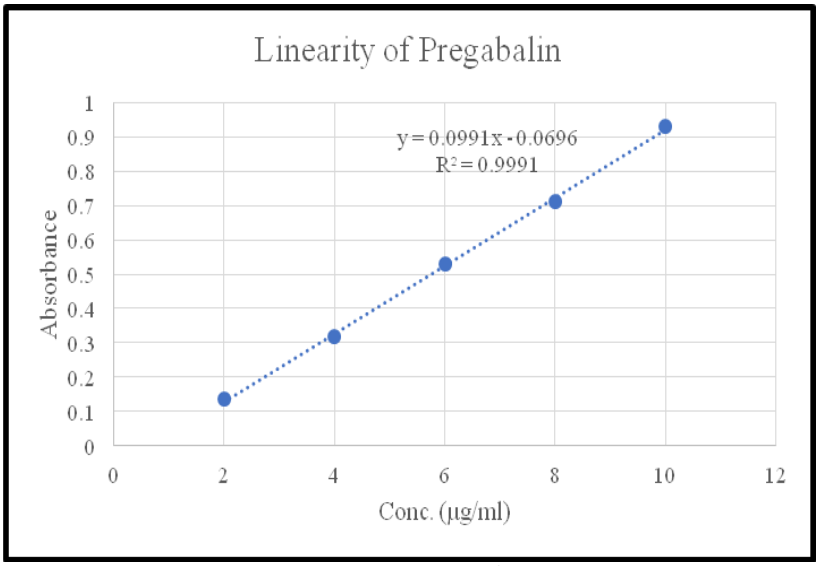


Figure 13: Calibration graph of Pregabalin

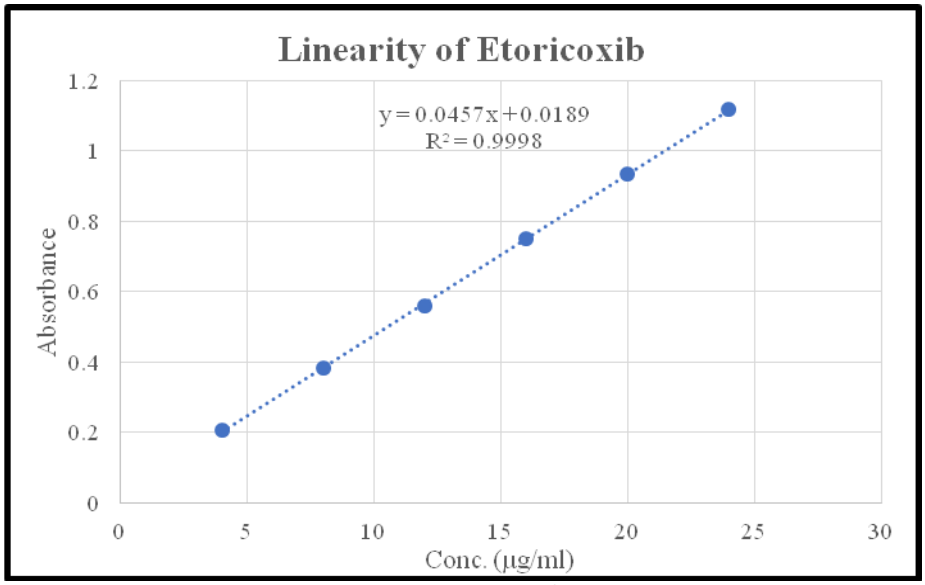


Figure 14: Calibration graph of Etoricoxib

Table 2: Statistical data for the regression equation of the proposed method

Parameter	Pregabalin	Etoricoxib
Analytical wavelength (nm)	576	284
Linearity range (µg/ml)	2-10	4-24
Regression equation	0.0991x-0.0696	0.0457x+0.0189
Slope	0.0994	0.0446
Intercept	0.0718	0.0196
Correlation coefficient (R^2)	0.9991	0.9998
Limit of detection (µg/ml)	0.0766	0.5674
Limit of Quantification (µg/ml)	0.2321	1.7193

2. Precision:

The precision of an analytical method expresses the closeness of agreement between a series of measurements which are obtained by performing multiple samplings of the same homogenous sample under the given conditions of the method. Here, the intra-day (Repeatability) and inter-day precision was determined. For that three-concentration having lower, upper and middle limits of both the drugs were taken and analysed three times on the same day for intra-day precision and on 3 different days for inter-day precision at the same concentration level. The % RSD was calculated (Table 3 and 4) and was found to be less than 2.

Table 3: Intraday and Interday precision of Pregabalin

	Conc.	Mean ± SD (n=3)	%RSD
Intra day	2	0.136±0.0012	0.882
	6	0.525±0.0036	0.686
	10	0.931±0.003155	0.333
Inter day	2	0.135±0.001	0.741
	6	0.525± 0.0035	0.667
	10	0.933±0.0031	0.327

Table 4: Intraday and Interday precision of Etoricoxib

	Conc.	Mean ± SD (n=3)	%RSD
Intra day	4	0.209±0.002	0.957
	12	0.550±0.0026	0.473
	20	0.911±0.0048	0.527
Inter day	4	0.211±0.0018	0.85
	12	0.549±0.0023	0.419
	20	0.909±0.0051	0.561

3. Accuracy:

The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analysed sample formulation at 80%, 100 % and 120% levels. The recovery studies were carried out and the percentage recovery and percentage relative standard deviation of the percentage recovery were calculated and given in Table 5.

Table 5: Accuracy data of Pregabalin and Etoricoxib

Drug	% Spiked	Conc. from formulation	Standard conc. added	Conc. recovered	%recovery ± SD (n=3)	% RSD
Pregabalin	80	7.5	6	6.11	101.83±0.46	0.452
	100	7.5	7.5	7.55	100.66±0.423	0.42
	120	7.5	9	9.06	100.64±0.501	0.498
etoricoxib	80	6	4.8	4.84	100.83±0.456	0.452
	100	6	6	5.89	98.16±0.412	0.42
	120	6	7.2	7.22	100.28±0.468	0.467

Analysis of the marketed formulation:

The assay of Pregabalin and Etoricoxib was found to be 99.77 and 99.96 % respectively which is within the acceptance criteria (98-102%).

Table 6: Assay results of the Marketed Formulation

Drug	Conc. (µg/ml)	Conc. Found (µg/ml)	% Recovery ± SD (n=6)	% RSD
Pregabalin	7.5	7.48	99.77 ± 0.436	0.437
Etoricoxib	6	5.998	99.96 ± 0.392	0.392

Estimation of Pregabalin using Smartphone application:

The linearity of the standard pregabalin was taken in the range of 2-10 µg/ml. The calibration curve and regression equation generated by the application was shown in the figure 16. The concentration of sample solution was found to be 7.443 µg/ml (Nominal 7.5 µg/ml). The % assay was found to be 99.24 % which is within the acceptance criteria.

Figure 15: Chart of colour intensity corresponding to the concentration of Pregabalin

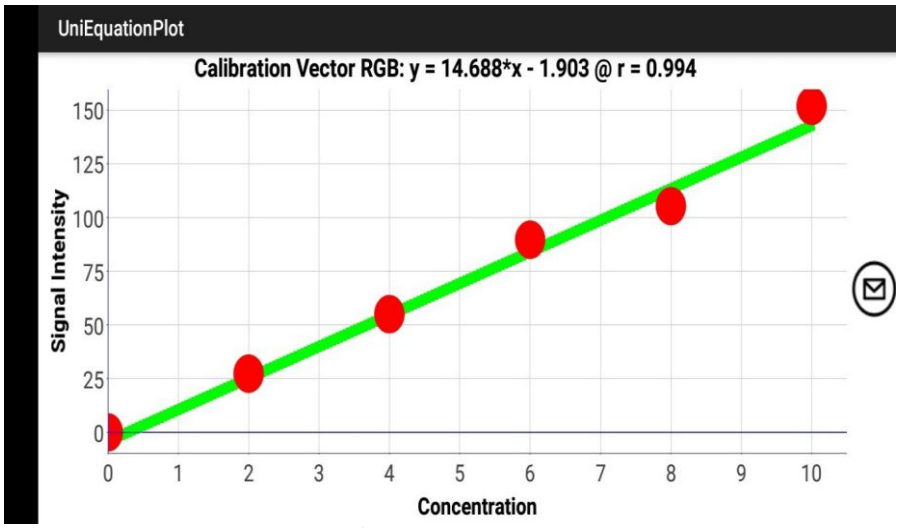


Figure 16: Calibration graph of Pregabalin generated by Photometrix Application

Comparison between UV-spectrophotometric method and Smartphone Image Analysis by T-Test:

Table 7: T-test table for the comparison of UV spectrophotometric and Smartphone Application method

	Variable 1	Variable 2
Mean	99.695	99.24
Variance	0.02587	0.28576
Observations	6	6
Pooled Variance	0.155815	
Hypothesized Mean Difference	0	
df	10	
t Stat	1.996491	
P(T<=t) one-tail	0.036909	
t Critical one-tail	1.812461	
P(T<=t) two-tail	0.073818	
t Critical two-tail	2.228139	

In order to compare two different methods statistically, the Student's t-test was used to analyse the results for the sample and the data are shown in the table 7.

The calculated t-value was smaller than the critical t-value, therefore showing no statistical difference at a 95% confidence level between UV spectrophotometric and Smartphone Image Analysis.

CONCLUSION

A simple, precise, accurate and sensitive method was developed for the

simultaneous estimation of Pregabalin and Etoricoxib. The method was validated according to the ICH guidelines. The method can be used for the routine analysis of the drugs in their pharmaceutical dosage form. Also, the developed colorimetric method was successfully extended for the estimation of Pregabalin using smart phone application. This application can be used as an alternative to sophisticated and high-cost devices in quantitative analysis.

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Conflict of Interest: None

Source of Funding: None

Ethical Approval: Approved

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