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Stability Indicating HPLC Method for Simultaneous Estimation of Pregabalin and Methylcobalamin from Combined Dosage Form

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ABSTRACT:

A stability indicating HPLC method has been described for the estimation of Pregabalin and Methylcobalamin in combined Dosage form using Agilent technologies 1290 series HPLC and Column Merck Purosphere encapped C_{18} column (250 X 4.6mm,5µm), in isocratic mode consisting of DAD detector with mobile phase Water :Methanol of(65: 35)%v/v at flow rate of 1 ml/min. The effluent is monitored at 205 nm. The peaks obtained were sharp having clear baseline with retention time 5 mins and 11 mins for Pregabalin and Methylcobalamin. The linearity observed in the range of 1125µg/ml to 4875µg/ml and the correlation co- efficient value of drug was found to be 0.999, which shows the good linearity between concentration of drug and response. The % C.V of precisions was less than 2, which indicate that the method has good degree of reproducibility. Degradation study performed using Acid, alkali & water hydrolysis, Oxidation, thermal, Photo and UV degradation in which degradation product separated in case of alkali, hydrolysis, Acid, Alkali, Photo and UV degradation and while in Humidity degradation no relative degradation was observed. Pregabalin and Methylcobalamin peaks is spectrally pure in each case and peak purity is obtained more than 990. For routine analysis, formulations containing Pregabalin and Methylcobalamin LOD, LOQ were found to be 12.11 µg /ml and 0.26 µg /ml, 36.69 µg /ml and 0.81 µg /ml respectively. Thus the proposed method is suitable.

KEY WORDS: Pregabalin, Methylcobalamin, HPLC Method, Degradation.

INTRODUCTION:

Pregabalin (PGB) is an anticonvulsant drug used in neuropathic pain also used as an adjunct therapy for partial seizures with or without secondary generalization in adults [1]. Pregabalin is S-enantiomer of 3-aminomethyl-5-methylhexanoic acid. Mechanism of action is thought to be linked to its high affinity α2d (alpha2delta) subunit of the voltage-dependent calcium channel in the central nervous system and decreases the release of neurotransmitters and reduce postsynaptic excitability [2]. Pregabalin does not bind to $GABA_a$ and $GABA_b$ receptor, not directly interfere the metabolism of GABA nevertheless it is supposed to mimic GABA. The maximum recommended dose of PGB is 100 mg to three times a day 300 mg per day in patient with creatinine clearance of at least 60 ml min^{-1} [3]. The chemical structure of pregabalin is shown in figure 1.

Methylcobalamin (MC) (mecobalamin, MeCbl, or MeB12) is a cobalamin, a form of vitamin B12, used in the treatment of megaloplastic aneamia, diabetic neuropathy and peripheral neuropathy. The Chemical formula of MC is $C_{63}H_{91}CoN_{13}O_{14}$ and IUPAC name is cobalt (3+) mecobalamin [4]. It is equivalent physiologically to vitamin B12 and can be used to prevent or treat pathology

arising from a lack of vitamin B12, such as pernicious anemia it is also used in the treatment of peripheral neuropathy, diabetic neuropathy, and as a preliminary treatment for amyotrophic lateral sclerosis [5,6]. Unlike the most common form of vitamin B12, cyanocobalamin, methycobalamin is required to protect against neurological diseases and aging [7]. The liver converts a small amount of cyanocobalamin into methylcobalamin but higher amounts are needed to actually correct neurological defects and prevent aging. High amounts of methylcobalamin are needed to regenerate neurons and myelin sheath that protects nerve axons and peripheral nerves. The chemical structure of MC shown in figure 2.

The review of literature revealed HPLC, HPTLC, LC and UPLC-MSMS Spectrophotometric methods are available for PGB and MC, but the Stability indicating assay methods for both of the drugs is not available [8,9,10,11]. Therefore the present study aims to develop accurate, simple and precise Stability indicating HPLC assay method for the analysis of PGB and MC in combined dosage as per the International conference on Harmonization (ICH) guidelines. This proposed method can be successfully employed for quality control during manufacture and for assessment of the stability of both drugs in bulk samples and their combined tablet dosage forms.

MATERIAL AND METHODS

Drug Substance

Pregabalin and Methylcobalamin gift sample and Marketed Formulation PREBEN were obtained from La-Renon Pvt. Ltd. The ratio of Pregabalin to Methylcobalamin was 1:10 respectively. HPLC grade methanol, NaOH, HCL, H_2O_2 was purchased from Merck and the water used was of Milli Q grade. All the glass apparatus used was of class A grade. All the solution were sonicated till drug dissolved and filtered through 0.45 µ filter.

Intrumentation

The HPLC instrument used was of Agilent Technologies 1290 with DAD detector. The Column used was Purosphere Merck – C18 Encapped (250mm×4.6mm, 5µm) with Chromatographic Software named EZ Chrome Digital. The pH and Electronic analytical balance used was of Metler Toledo. Membrane filter paper used was of 0.45 µm Nylon 66 (N66) 47 mm.

Preparation of Solutions

Preparation of Diluted Standard solution of PGB of 1875 µg/ml

375 mg of PGB was transferred to a 50 ml volumetric flask (7500 µg/ml). From the above Standard stock solution, 25 ml of aliquot was transferred into 100 ml volumetric flask and volume was adjusted to the mark with Water to produce diluted solution of 1875 µg/ml.

Preparation of Diluted Standard solution of MC of 18.75 µg/ml

75 mg of MC was transferred to a 200 ml volumetric flask. From that 5ml of aliquot was transferred into 100ml volumetric flask and make up to the mark to make a final solution of 18.75 µg/ml.

Preparation of Sample Stock Solution (PREBEN Capsule)

690 mg of capsule powder (Equivalent weight taken from 20 capsules) was transferred to a 100 ml volumetric flask and diluted upto the mark with water then filtered through 0.45µ nylon filter.

Selection of wavelength for simultaneous estimation:

Standard Solution of PRG 1875 µg/ml and MC 18.75 µg/ml used for the selection of wavelength which were scanned between 200-400 nm in a Shimadzu UV-1800 UV-visible Double beams Spectrophotometer at a medium scanning speed.

Chromatographic Condition

Merck Purosphere encapped C_{18} column (250mm X 4.6mm,5µm), in isocratic mode consisting of DAD detector with mobile phase Water :Methanol 65: 35 %v/v at flow rate of 1 ml/min. Column was saturated by mobile phase for 30 mins by applying check pressure. When the system was saturated the effluent was monitored at 30 ^{o}C and 205 nm wavelength. The peaks obtained were sharp having clear baseline with retention time 5 mins and 11 mins for Pregabalin and Methylcobalamin. A number of aliquots were prepared from stock solution to get concentration of 1125 to 4875 µg/ml and 11.25 to 48.75 µg/ml for PGB and MC respectively were run for 6 times. The peak area as well as the run time was kept in check for both of the drugs. Linearity was calculated by taking average peak area on Y-axis and concentration on X-axis separately for PGB and MC.

Preparation of Calibration Curves

Calibration curve was prepared by taking appropriate aliquots of standard Pregabalin and Methylcobalamin stock solutions in different 50ml volumetric flasks and diluted up to the mark to get a concentration in the range of 1125 to 4875 µg/ml for Pregabalin and 11.25 to 48.75 µg/ml for Methylcobalamin. The calibration curves of Pregabalin and Methylcobalamin was constructed by plotting average peak area versus % of concentration.

Method Validation

The analytical method was validated for various parameters as per ICH guidelines.

Linearity

The linearity of the method was determined in concentration range of 1125-4875 mcg/ml for PGB and 11.25-48.75 mcg/ml for MC. Each solution was injected in triplicate. The average peak area versus concentration data of both drugs was treated by least squares linear regression analysis. Linearity was checked over the same concentration range on three consecutive days.

Specificity and Selectivity

Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by examining blank matrix samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other excipients. Two different samples were injected and studied with respective excipients. The HPLC chromatograms recorded for the drug matrix showed almost didn't interfered peaks within retention time ranges. Thus, the HPLC method proposed in this study was selective.

Accuracy and Recovery

Accuracy was evaluated in triplicate, at three different concentrations equivalent to 80, 100, and 120% of the target concentration of active ingredient, % of recovery was then calculated.

Precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. Precision of the method was determined with sample stock solution. The precision of the method was verified by repeatability (intraday) and the intermediate precision studies. Repeatability studies were performed six times by analysis of the concentrations of sample stock solution of PGB and MC. Intermediate precision was performed by performing the same procedure on the same day for intra-day precision. The intermediate precision (Interday) of the method was checked by performing same procedure on different days under same experimental conditions. The sample application and measurement of peak area were expressed in terms of relative standard deviation (%RSD)

Limit of Detection (LOD)

The limit of detection in the smallest concentration can be detected and not quantified as an exact value. LOD can be calculated as

$LOD = 3.3\ \sigma/S$

Where σ= Standard deviation of the y-intercept, S=Slope of calibration curve.

Limit of Quantification (LOQ)

The limit of the quantification is the lowest amount of analyte in the sample which can be determined quantitatively.

$LOQ=10\ \sigma/S$

Where σ= Standard deviation of the y-intercept, S=Slope of calibration curve.

Robustness

Variation in flow rate and temperature was made to the analytical method in order to evaluate and measure the capacity to remain unaffected by such variations. During robustness testing two conditions were varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (± 0.2 ml/min) and change in mobile phase composition.

System Suitability Parameters [12]

The results obtained by doing the assay of marketed formulations was optimized and validated as per the International conference on Harmonization (ICH) guidelines like plate number (N), tailing factor (K),

resolution and relative retention time of samples. For assessing system suitability, six replicates of working standards samples of PGB and MC were injected.

Degradation Sample Preparation

20 capsule powders were weighed and transferred into petridish. Weight equivalent to 100 mg was taken (75mg PGB and 0.75mg MC) of powdered sample into a 100ml volumetric flask dissolved and diluted to volume with HPLC grade water and filtered the solution using 0.45µ Nylon filter.

Acid Hydrolysis

Transferred 1ml of above stock solution to10ml volumetric flask and added 1ml of 0.1N HCL and refluxed for 30min at 60°C. Cooled to room temperature and neutralized using 1ml of 0.1N NaOH and volume was made with HPLC grade water.

Base Hydrolysis

Transferred 1ml of above stock solution to10ml volumetric flask and added 1ml of 0.1N NaOH and reflux for 45 min at 60°C. Cooled it to room temperature and neutralized using 1ml of 0.1N HCl and volume was made with HPLC grade water.

Peroxide Hydrolysis

Transferred 1ml of above stock solution to10ml volumetric flask and added 1ml of 3%v/v of H_2O_2 and refluxed for 30min at 60°C. Cooled to room temperature and volume was made with HPLC grade water.

Thermal Degradation

20 capsules powder was taken and transferred 200mg (150mg PGB and 1.5mg MC) powder into petridish. Sample was heated in oven for about 8 hrs at 105°C. From this accurately weighed 100 mg of powdered sample and transferred into a 100ml volumetric flask, dissolved and diluted to volume with HPLC grade water. 1ml of above stock solution was transferred into10ml volumetric flask and filtered using 0.45µ Nylon filter.

Photolytic Degradation

Photolytic degradation study was carried out by exposing the accurately weighed 200mg (150mg PGB and 1.5mg MC) of capsule powder to UV light in a photolytic chamber at 1.2 million lux for 24 hr, After 24hrs weighed accurately 100 mg of powdered sample into a 100ml volumetric flask, dissolved and diluted to volume with HPLC grade water upto the mark. 1ml of above stock solution transferred into10ml volumetric flask and filter the solution using 0.45µ Nylon filter.

Humidity

Humidity degradation study was carried out by exposing 200mg (150mg PGB and 1.5mg MC) of capsule powder to 75% RH in Stability Chamber for 7 days, After 7 days 100 mg of above powdered sample taken into a 100ml volumetric flask, dissolved and diluted to volume with HPLC grade water up to the mark. 1ml of above stock solution transferred into10ml volumetric flask and filter through 0.45µ Nylon filter.

The chromatograms of PGB and MC, after being subjected to different degradation conditions, were compared with blank solutions injected in a similar manner and with recently prepared solutions.

RESULT AND DISCUSSION

Optimized Chromatographic condition

There are many methods developed using Different types of columns. Most of the methods have complex mobile phases hence trials were performed to develop method which is simple and results in good resolution among the drugs and the degraded products. These changes included change in mobile phase composition in isocratic elution as well as gradient modes on different C18 columns.

The optimized chromatographic conditions is shown in Table 1. The best peak shape and maximum separation was achieved with mobile phase composition of water: Methanol (65:35)%v/v. The best separation, peak symmetry and reproducibility were obtained on Purosphere Merck encapped C18 (250mm×4.6mm I.D; 5 µm).Optimum wavelength was found 205 nm. And flow rate for optimized condition was kept 1ml/min.

METHOD VALIDATION

Linearity, LOD and LOQ

The Calibration plot figure 3(a) & (b) showed linearity over the concentration range (1125-4875) µg/ml and (11.25-48.75) µg/ml for PGB and MC respectively. The relative standard deviation was found <2%. The regression results

indicate that method was linear in the concentration range.

The LOD was found to be 12.11 µg/ml and 0.26 µg/ml and LOQ were found to be 36.69µg/ml and 0.81µg/ml for PGB and MC respectively.

The Linearity overlain spectra are depicted in figure 4(a) & (b). The spectra were obtained from lower concentration to higher concentration linearity range and run time for PGB was approx. 4.9 min and for MC approx. 10.9 min.

Accuracy and Precision

Accuracy studies are show in Table 2 (a) & (b) for PGB and MC respectively. This result reveals that the Percentage recovery is obtained in the range of 98-101% for both the drugs. The value of standard deviation is less than 1% indicating that the proposed method is accurate for the simultaneous estimation of both drugs from their combination dosage form.

Repeatability

The Repeatability was performed by the standard working solution of PGB and MC. Six Replicates of solution were taken. The %Relative Standard Deviation was less than 1%.the results are show in Table 3

Intra-day and inter-day

The precision was performed on the same day for intra-day. The results are shown in Table 4(a). The inter-day precision was carried out on different days and in different conditions. The %Relative Standard Deviation was found less than 2%.The inter-day data are show in Table 4(b).

Robustness

Table 5 (a) & (b) shows that variation in flow rate and in composition of mobile phase were not affecting the method developed. The %RSD obtained are less than 2%. Hence this method is said to be robust.

System Suitability Parameter

The table 6 reveals that the retention time obtained were at mean 4.9 and 10.9min for PGB and MC respectively. For system suitability Tailing Factor should be less than 1%, theoretical plates should be more than 1000 and resolution should be less than 2%. Hence the system is suitable to use for Stability indicating assay method.

Overview of forced degradation

Stress-degradation studies of the drug substances can help identifying the possible degradation products which can in turn help establishing the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating analytical procedures used. The chromatograms of PGB and MC, after being subjected to different degradation conditions, were compared with blank solutions injected in a similar manner and with recently prepared solutions. This peak purity showed the specificity of the developed method clearly. The study was conducted to show the non interference of any degraded peak and not to identify degradation products.

Acid degradation data (figure 5) shows that at RT 3.23 min and RT 9.10 min degraded peaks may be present for PGB and MC, the degradation found was 3% and 17% respectively.

Base degradation data (figure 6) reveals that at RT 3.29 and RT 9.01 degraded peaks were seen, the % degradation was found to be 8% and 15% for PGB and MC respectively.

For the oxidation data (figure 7) peaks obtained at RT 2.72 is of H_2O_2 and at RT 3.30 and RT 9.15 may be degraded peak with % degradation 8% and 22% of PGB and MC respectively.

Thermal degradation shows (figure 8) that RT 3.65min and RT 8.93min unknown peaks were obtained which may be degraded peaks with % degradation 19% and 20% of PGB and MC respectively.

In humidity degradation data (figure 9) there was no change observed. And for Photodegradation 10% degradation was observed for MC (figure 10). The results from forced degradation studies were summarized in Table 7.

CONCLUSION

Stress testing (or forced degradation studies) is an important part of drug development process and the pharmaceutical industry has much interest in this area. A simple, rapid, accurate and precise stability-indicating HPLC analytical method has been developed and validated

for the quantitative analysis of PGB and MC in combined dosage forms. The results of stress testing were undertaken according to the ICH guidelines revealed that the method is specific and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products in dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies. Also this method is Applicable to industrial Q.A department.

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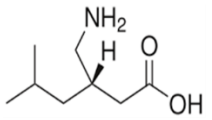

Figure 1 Structure of Pregabalin

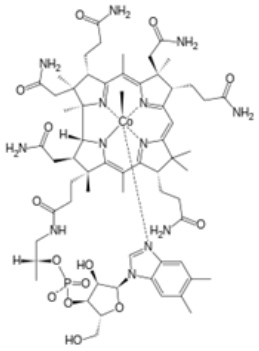

Figure 2 Structure of Methylcobalamin

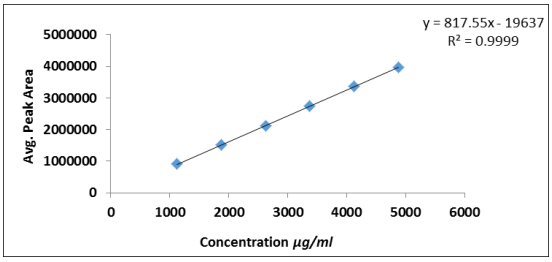

Figure 3(a) Linearity curve of PGB

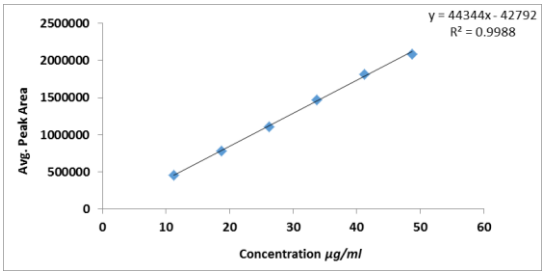

Figure 3(b) Linearity curve of MC

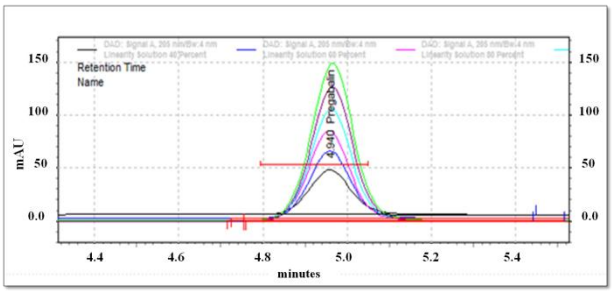

Figure 4(a) Linearity overlain spectra of PGB

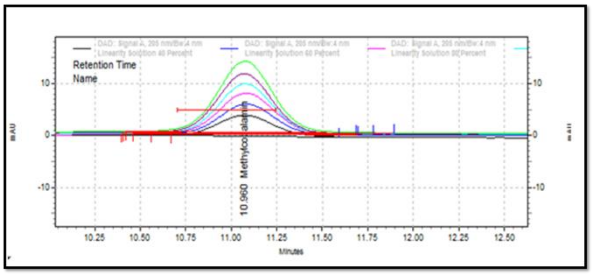

Figure 4(b) Linearity overlain spectra of MC

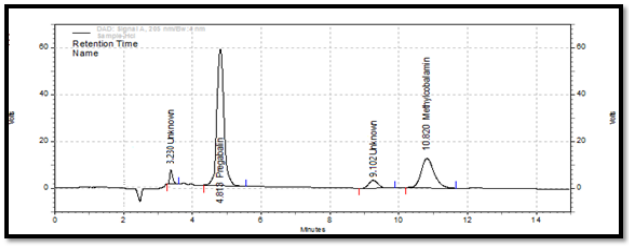

Figure 5 Chromatogram of acid degradation of PGB(1875µg/ml) and MC(18.75µg/ml) at 205 nm

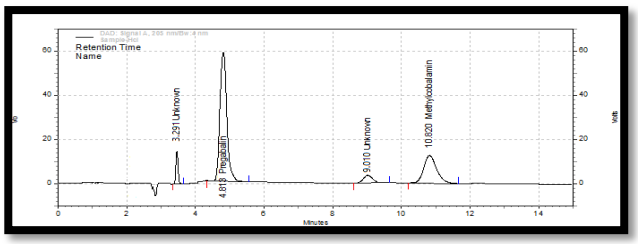

Figure 6 Chromatogram of base degradation of PGB(1875µg/ml) and MC(18.75µg/ml) at 205 nm

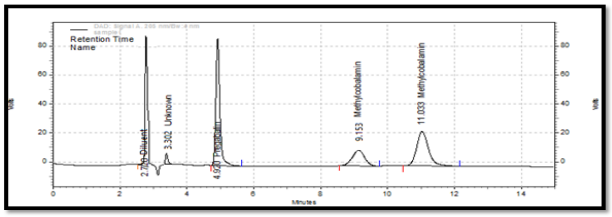

Figure 7 Chromatogram of peroxide degradation of PGB(1875µg/ml) and MC(18.75µg/ml) at 205 nm

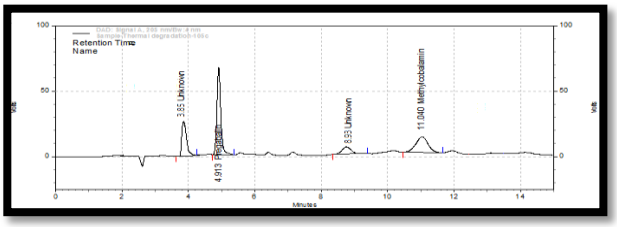

Figure 8 Chromatogram of thermal degradation of PGB(1875µg/ml) and MC(18.75µg/ml) at 205 nm

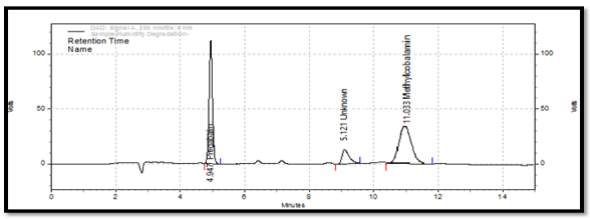

Figure 9 Chromatogram of photo degradation of PGB (1875µg/ml) and MC(18.75µg/ml) at 205 nm

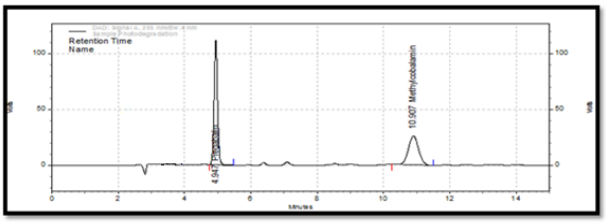

Figure 10 Chromatogram of Humidity degradation of PGB(1875µg/ml) and MC(18.75µg/ml) at 205nm

Table 1 Optimized Chromatographic Conditions

Stationary phase	**Merck Purosphere encapped C_{18} column (250 X 4.6mm, 5µm)**
Flow rate	1.0 ml/min
Isocratic program	Water: Methanol (65:35%v/v)
Diluent	Water
Column temperature	30˚C
Injection volume	10 µl
Wavelength	205 nm

Table 2(a) Accuracy Study for of PGB

Amt of PGB taken (µg)	Level	Amt of std PGB added (µg)	Total Amt of PGB (µg)	Amt of PGB recovered (µg)	Mean Recovery(%) ± SD
1125	80%	900	2025	885.44	99.84 ± 0.39
1125	100%	1125	2250	1120.97	100.32 ± 0.23
1125	120%	1350	2475	1351.97	100.54 ± 0.36

Table 2(b) Accuracy Study for of MC

Amt of MC taken (µg)	Level	Amt of std MC added (µg)	Total Amt of MC (µg)	Amt of MC recovered (µg)	Mean % Recovery ± SD
11.25	80%	9	20.25	8.70	98.39 **±** 0.34
11.25	100%	11.25	22.50	11.25	99.80 **±** 0.28
11.25	120%	13.50	24.75	13.64	100.82 **±** 0.21

Table 3 Repeatability data for PGB and MC (n= 6)+

Conc. of PGB (µg/ml)	Area	Conc. of MC (µg/ml)	Area
1875	1568944	18.75	787782
	1569875		781374
	1569836		785457
	1561387		788195
	1570258		781895
	1597965		785392
Mean	1568044	**Mean**	785015.8
SD	3364.45	**SD**	2866.96
% CV	0.22	**% CV**	0.365

Table 4(a) Data for intraday precision for PGB and MC (n=3)

PGB			**MC**		
Conc. (µg/ml)	**Mean area ±SD**	**% CV**	**Conc. (µg/ml)**	**Mean area ±SD**	**% CV**
1875	1513861 **±** 6644.48	0.44	18.75	785150.3 **±** 2319	0.30
2625	2123841 **±** 5796.62	0.27	26.25	1125916 **±** 3452	0.31
3375	2728715 **±** 4610.2	0.17	33.75	1457220 **±** 3028	0.21

Table 4(b) Data for interday precision for PGB and MC (n=3)

PGB			MC		
Conc. (µg/ml)	**Mean area ±SD**	**% CV**	**Conc. (µg/ml)**	**Mean area ±SD**	**% CV**
1875	1527194 ± 11038	0.72	18.75	784817 **±** 4333	0.55
2625	2133841 ± 10173	0.47	26.25	1132250 **±** 7154	0.63
3375	2741715 ± 15464	0.56	33.75	1457220 **±** 1226	0.84

Table 5(a) Change in flow rate of mobile phase – Data of Sample preparation

Standard	Area of PGB		Area of MC	
	0.8ml/min	**1.2ml/min**	**0.8ml/min**	**1.2ml/min**
Inj-1	1510997	1510756	779829	772325
Inj-2	1514532	1506025	786889	776956
Inj-3	1523421	1529176	785367	780234
Inj-4	1533267	1499643	787423	789887
Inj-5	1546789	1502506	780922	798056
Mean area	1525801 ± 14560	1509621 ± 11691	784086 ± 3491	783492 ± 10380
%RSD	0.95	0.774492	0.45	1.32

Table 5(b) Change in mobile phase Composition

Standard	Area of PGB		Area of MC	
Water: Methanol	67:33	63:37	67;33	63:37
Inj-1	1510435	1501935	783736	781901
Inj-2	1544532	1501135	775165	789899
Inj-3	1516335	1500335	787015	781156
Inj-4	1521335	1510035	781575	771589
Inj-5	1513935	1519535	789382	771853
Mean area	1523185 ± 12974	1505652 ± 7703	784402 ± 5509	780332 ± 7358
%RSD	0.85	0.51	0.70	0.94

Table 6 System suitability parameters

Parameter	PGB	MC
Retention time(min)± % CV (n=5)	4.91 ± 0.71	10.93 ± 0.23
Tailing factor ± % CV (n=5)	0.98 ± 1.3	1.18 ± 1.28
Theoretical plates ± % CV (n=5)	3147.40 ± 0.77	3572.40 ± 1.07
Resolution(<2%)	1.23	

Table 7 % Degradation and peak purity index of PGB and MC

Degradation condition	% Degradation		Peak purity index	
	PGB	**MC**	**PGB**	**MC**
Acid	3	17	0.9999	0.9990
Base	8	15	0.9998	0.9996
Peroxide	8	22	0.9995	0.9996
Thermal	19	20	0.9996	0.9991
Photo	-	10	0.9998	0.9990
Humidity	-	-	0.9998	0.9998