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# Simultaneous Estimation of Pregabalin and Methylcobalamine in Pharmaceutical Formulation by HPTLC-Densitometry Method

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## Abstract

A simple, precise, rapid, selective, and economic reversed phase high-performance thin layer chromatography (HPTLC) method has been established for simultaneous analysis of PRG and MC. HPTLC method was developed using on precoated silica gel $F_{254}$ G60 plates as stationary phase, using methanol:toluene:ammonia (30%) (8:2:0.4 v/v/v) as mobile phase. The plates were scanned at approximately 497 nm for both PRG and MC respectively. In HPTLC method both the drugs were resolved using proposed mobile phase and $R_f$ value was found to be 0.60 for PRG and $R_f$ 0.33 for MC. The method was found to linear in the range 1500-7500 ng/band for PRG, 150-750 ng/band based for MC respectively. This HPTLC procedure is economic, sensitive, and less time consuming than other chromatographic procedures. It is important tool for analysis of combined dosage form.

**Keywords:** HPTLC; Precoated silica gel $F_{254}$ G60; Pregabaline (PRG); Methylcobolamine (MC)

## Introduction

Pregabaline (PRG; (3S)-3-(aminomethyl)-5-methylhexanoic acid; (Figure 1a) is a widely used Anticonvulsant and Analgesic for Epilepsy [1]. Methylcobalamine (MC; carbanide;cobalt;[5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2-(hydroxymethyl) oxolan-3-yl]1-[3-[2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19- octamethyl-2,7,12,17-tetrahydrocorrin-3-yl]propanoylamino]propan-2-yl hydrogen phosphate (Figure 1b) is Derivative form of vitamin B12. It is used for the treatment of peripheral neuropathy, diabetic neuropathy.

Literature survey revealed that various analytical methods like HPLC [2-5], UV [6-8] and HPTLC [9] have been reported for the determination of PRG and MC either individually or combination with some other drugs. The review of literature prompted us to develop an accurate, selective and precise simultaneous method for the estimation of PRG and MC in combined dosage forms.

## Experimental

### Chemicals and materials

PRG was procured from Torrent Pharmaceuticals Ltd, Ahemdabad and Methylcobalamine was obtained from sajal enterprises. Methanol (HPLC Grade), Triethylamine (AR Grade), Ninhydrin (AR Grade), Isopropylalcohol (AR Grade), Ammonia and n-Butanol were used as solvents to prepare the mobile phase. All the reagents used were of Analytical reagent grade and used without further purification. Capsule formulation PREGABALIN M 75 (Torrent Pharmaceuticals Ltd) and NOVA PLUS CAP (Cipla Pharmaceuticals Ltd.) containing labeled amount of 75 mg Pregabalin and 0.75 mg of Methylcobalamine was procured from local market (Table 5).

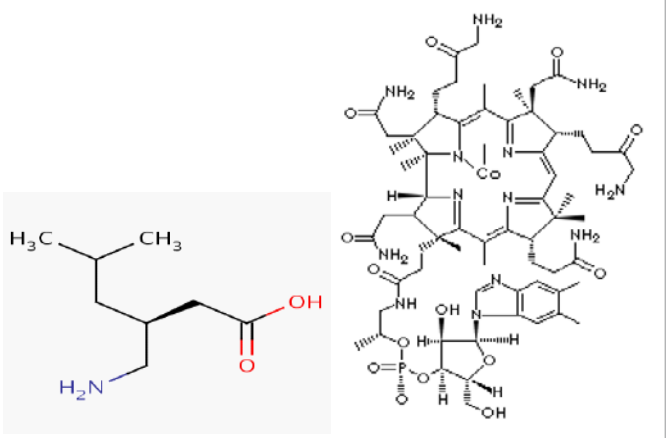


**Figure 1:** Structure of pregabalin (a) and methylcobalamine (b).

### Chromatographic conditions

A Pre-coated silica gel G60–F254 aluminum sheet (100×100 mm, thickness layer 0.2 mm) pre washed with methanol was used as stationary phase. The linear ascending development was carried out in a CAMAG twin-trough glass chamber (20×20 cm) equilibrated with the mobile phase methanol:toluene:ammonia (30%) (8:2:0.4, v/v/v, (pH 7.5) for 20 min at room temperature. The length of the chromatogram run was 70 mm. Quantitative evaluation of the plate was performed in absorbance mode at 497 nm. The slit dimensions were 5 mm length and 0.45 mm width, with a scanning rate of 20 mm/s with a computerized CAMAG TLC scanner -3 integrated with win CATS 4 software.

### Sample preparation

Twenty Capsules were taken and drug content was collect by empty gelatin shell. Capsule powder equivalent to 0.75 gm of PRG (0.0075 gm of MC) was accurately weighed and transferred to a 100 mL volumetric flask. A few mL of methanol was added to the above flask and flask was sonicated for 10 min. The solution was filtered using Whatman filter

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paper No.1 in another 100 mL volumetric flask and volume was diluted to the mark with the methanol. One ml aliquot was taken from above solution to a 10 ml volumetric flask. 1 ml aliquot of standard stock solution (750 ng/band) of MC was added to the same flask and volume was making up to the mark with methanol to obtain final concentration of 750 ng/band of PRG (and 82.5 ng/band of MC). Appropriate aliquot of these solutions were applied to HPTLC plates and analyzed for PRG and MC content using the proposed method as described earlier. From the developed chromatogram spot area and $R_f$ values were determined.

**Preparation of standard solution:** MC (7.5 mg) was accurately weighed and transferred to 10 ml volumetric flasks and dissolved in few ml methanol. The volume was made up to the mark with methanol to yield a solution containing 750 µg/ml MC. PRG (7.5 mg) was accurately weighed and transferred to 10 ml volumetric flask and dissolved in few ml methanol. 1 ml of aliquot stock solution of MC was added to this flask and volume was made up to mark with methanol to yield a solution containing 75 µg/ml MC and 750 µg/ml PRG. This mixture was used for the further study.

## Method validation

The developed method was validated for linearity and range, specificity, accuracy, precision, Limit of detection, Limit of quantitation, robustness and solution stability as per ICH guidelines.

**Linearity and range:** Linearity of the method was evaluated by constructing calibration curves at five concentration levels over a range of 1500-7500 ng/band and 150-750 ng/band of PRG and MC respectively. The calibration curves were developed by plotting peak area versus concentration with the help of the win CATS software (Figure 2).

**Specificity:** The specificity of the method was ascertained by analyzing PRG and MC in presence of excipients like magnesium stearate, talc and micro crystalline cellulose commonly used for capsule formulations. The bands of PRG and MC were confirmed by comparing $R_f$ values and respective spectra of sample with those of standards. The peak purity of PRG and MC was assured by comparing the spectra at three different levels, that is, peak start, peak apex and peak end positions (Table 1).

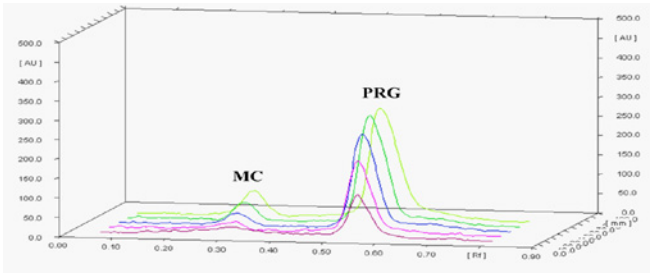


**Figure 2:** Three dimensional overlay of HPTLC densitograms of calibration bands of PRG and MC.

| Sample | Correlation of center and slope spectra | |
|---|---|---|
| | r (s, m) | r (m, e) |
| PRG | 0.994 | 0.991 |
| PRG (PREGALIN M 75) | 0.991 | 0.994 |
| MC | 0.994 | 0.992 |
| MC (PREGALIN M 75) | 0.997 | 0.996 |

**Table 1:** Peak purity correlation results of PRG and MC in two formulations at peak start, middle and end using WINCATS software.

**Accuracy (%Recovery):** The accuracy of the method was determined by calculating recoveries of PRG and MC by method of standard additions. Known amount of PRG (50%, 100%, 150%) and MC (50%, 100%, 150%) were added to a pre quantified sample solution, and the amount of PRG and MC were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

**Method precision (Repeatability):** Repeatability of measurement of peak area was determined by analyzing PRG and MC sample (4500 and 450 ng/band) six times without changing the position of plate.

**Intermediate precision (Reproducibility):** The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as %RSD. Precision was evaluated in terms of intraday and interday precisions. Intraday precision was determined by analyzing sample solutions of PRG (3000, 4500, 6000 ng/band) and MC (300, 450, 600 ng/band) at three levels covering low, medium, and high concentrations of the calibration curve three times on the same day (n=3). Interday precision was determined by analyzing sample solutions of PRG (3000, 4500, 6000 ng/band) and MC (300, 450, 600 ng/band) at three levels covering low, medium, and high concentrations over a period of 3 days (n=3). The peak areas obtained were used to calculate mean and %RSD values (Figures 3-5).

**Limits of detection (LOD) and Limits of quantitation (LOQ):** The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines. LOD=3.3×σ/S; LOQ=10×σ/S; Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

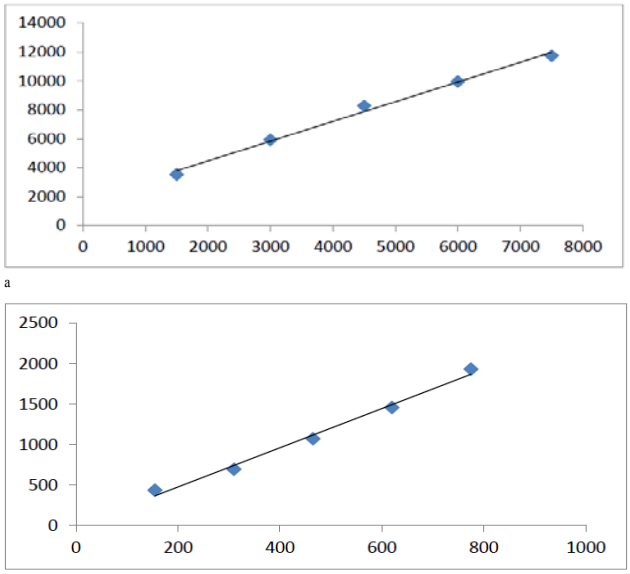


**Figure 3:** Calibration plot of PRG (a) and MC (b) by the developed HPTLC method.

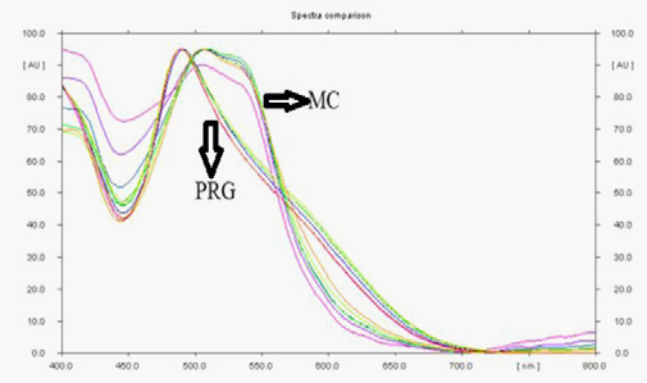


**Figure 4:** Overlain Absorbance spectrum of PRG and MC respectively.

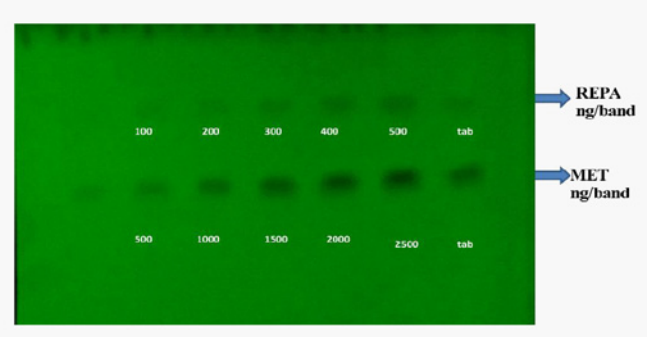


**Figure 5:** Photograph of developed HPTLC plate of MET and REPA.

**Robustness:** Small changes in the chamber saturation time, solvent migration distance was introduced and the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 4500 ng/band and 450 ng/band of PRG and MC respectively. The mean and %RSD of peak areas were calculated.

**Solution stability:** The solutions at analytical concentration PRG (50, 200, 300 ng/band) and MC (0.5, 10, 20 ng/band) were prepared and stored at room temperature for 24 h and analyzed at interval of 0, 6, 12 and 24 h for the presence of any band other than that of PRG and MC and the results were simultaneously compared with the freshly prepared PRG and MC standard solution of the same concentration in the form of change in %RSD of the response obtained.

## Application of validated method to pharmaceutical formulation

Twenty Capsules were taken and drug content was collect by empty gelatin shell. Capsule powder equivalent to 0.75 gm of PRG (0.0075 gm of MC) was accurately weighed and transferred to a 100 mL volumetric flask. A few mL of methanol was added to the above flask and flask was sonicated for 10 min. The solution was filtered using Whatman filter paper No.1 in another 100 mL volumetric flask and volume was diluted to the mark with the methanol. One ml aliquot was taken from above solution to a 10 ml volumetric flask. 1 ml aliquot of standard stock solution (750 ng/band) of MC was added to the same flask and volume was making up to the mark with methanol to obtain final concentration of 750 ng/band of PRG (and 82.5 ng/band of MC). Appropriate aliquot of these solutions were applied to HPTLC plates and analyzed for PRG and MC content using the proposed method as described earlier. From the developed chromatogram spot area and Rf values were determined.

## Results and Discussion

### Method development and optimization of chromatographic conditions

A mobile phase consisting of methanol:toluene:ammonia (30%) (8:2:0.4, v/v/v) gave good separation of PRG and MC from its matrix. It was also observed that chamber saturation time and solvent migration distance were crucial in the chromatographic separation as chamber saturation time of less than 20 min and solvent migration distances greater than 70 mm resulted in diffusion of the analyte band. Therefore, methanol:toluene:ammonia (30%) (8:2:0.4 v/v/v) mobile phase with linear ascending development was carried out in a twin-trough glass chamber equilibrated with the mobile phase vapour for 20 min at room temperature. After development, the HPTLC plates were dried completely. These chromatographic conditions produced a well-defined, compact band of PRG and MC with optimum migration at $R_f$ 0.60 ± 0.006 and 0.33 ± 0.005 respectively. A mixed solution was filled in the syringe and under nitrogen stream; it was applied in form of band having concentration of 1500-7500 ng/band and 150-750 ng/band of PRG and MC respectively on a single plate. Plate was developed using methanol:toluene:ammonia (30%) (8:2:0.4 v/v/v) at room temperature, dried in air. After drying it was sprayed by ninhydrin (0.2% in isopropyl alcohol) and heated at 110°C for 2 min. Developed plate was subjected to densitometric measurements in scanning mode in the visible region of 400-800 nm and the overlain spectrum was recorded using Camag TLC Scanner 3. The overlain spectra showed that both the drugs absorbs appreciably at 497 nm so, it was selected for the densitometric analysis.

### Validation of the method

**Linearity:** The method was found to be linear in a concentration range of 1500-7500 ng/band and 150-750 ng/band of PRG and MC respectively, (n=5) with a co-relation coefficient of 0.9933 and 0.9950 of PRG and MC respectively. The regression data showed good linear relationship over the concentration range studied, demonstrating the suitability of the method for analysis.

**Specificity:** The specificity study was carried out to check the interference from the excipients used like microcrystalline cellulose, magnesium stearate and talc in the formulations by preparing synthetic mixture containing both the drugs and excipients. The chromatogram showed peaks for both the drugs without any interfering peak and the recoveries of both the drugs were above 98%.

**Accuracy:** Accuracy of an analytical method is the closeness of test results to the true value. It was determined by the application of analytical procedure to recovery studies, where a known amount of standard is spiked into pre analyzed samples solutions. Percentage recovery was found to be 99.86-100.24% and 97.83-99.54% for PRG and MC respectively. Recovery values demonstrated the accuracy of the method in the desired range (Table 2).

**Precision:** The %RSD value were found to be 0.15-0.28 of PRG and 0.82-1.81 of MC for intraday and 0.29-0.65 of PRG and 0.48-1.21 of MC for interlay. In all instances, %RSD values were less than 2%, confirming the precision of the method. Repeatability of the scanning device was studied by applying and analyzing PRG and MC sample (4500 and 450 ng/band) three times. RSD was less than 2% which was well below the instrumental specifications (Table 3).

**Limits of detection (LOD) and Limits of quantification (LOQ):** Under the experimental conditions used, the lowest amount of drug

| Amount of sample Taken (ng/band) | | Amount of standard drug added (ng/band) | | Standard addition (ng/band) | Amount of drug recovered (ng/band) | | % recovery ± % RSD (n=3) | |
|---|---|---|---|---|---|---|---|---|
| PRG | MC | PRG | MC | MC | PRG | MC | PRG | MC |
| 1500 | 15 | 0 | 0 | 150 | 1498.01 | 162.80 | 99.86 ± 1.56 | 98.66 ± 0.91 |
| 1500 | 15 | 750 | 7.5 | 150 | 2268.40 | 171.71 | 99.92 ± 0.98 | 99.54 ± 1.17 |
| 1500 | 15 | 1500 | 15 | 150 | 3007.44 | 176.10 | 100.24 ± 1.29 | 97.83 ± 0.97 |
| 1500 | 15 | 2250 | 22.5 | 150 | 3749.10 | 184.46 | 99.97 ± 1.37 | 98.38 ± 0.67 |

**Table 2:** Results from accuracy study.

| Parameters | PRG | MC |
|---|---|---|
| Range (ng/band) | 1500-7500 | 150-750 |
| Retention time (min) | 6.4 | 7.9 |
| Resolution | 2.02 | |
| Detection limit (ng/band) | 177 | 27 |
| Quantitation limit (ng/band) | 535 | 82 |
| Accuracy (%) | 99.86-100.24% | 97.83-99.54% |
| | Precision (%RSD) | |
| Intra-day (n=3) | 0.15-0.28 | 0.82-1.81 |
| Inter-day (n=3) | 0.29-0.65 | 0.48-1.21 |
| Instrument precision (%RSD) | 0.51% | 1.32% |
| Specificity | Specific | Specific |

**Table 3:** Summary of validation parameters of developed HPTLC method.

| Parameter | Method condition | % rsd of peak area | |
|---|---|---|---|
| | | PRG | MC |
| Chamber saturation Time (± 20) | 16 min | 1.41 | 1.97 |
| | 24 min | 1.10 | 1.44 |
| Development distance from spot application (± 10) | 7.7 cm | 1.04 | 2.02 |
| | 6.3 cm | 0.83 | 1.80 |

**Table 4:** Results from the robustness study of method.

| Formulation | Label claim (mg) | | Amount found | | % of lable claim (n=5) ± % RSD (n=5) | |
|---|---|---|---|---|---|---|
| | PRG | MC | PRG | MC | PRG | MC |
| PREGALIN M 75 | 75 | 0.75 | 74.50 | 0.738 | 101.26 ± 1.02 | 98.47 ± 0.63 |
| NOVA PLUS CAP. | 75 | 0.75 | 73.68 | 0.733 | 99.42 ± 0.74 | 97.29 ± 1.37 |

Capsule formulation PREGABALIN M 75 (Torrent Pharmaceuticals Ltd) and NOVA PLUS CAP (Cipla Pharmaceuticals Ltd.) containing labelled amount of 75 mg Pregabalin and 0.75 mg of Methylcobalamine n=number of determinations

**Table 5:** Results from analysis of pregabalin and methylcobalamine in the combined capsule dosage form.

that could be detected (LOD) for PRG and MC were found to be 177 and 27 ng/band respectively. The limit of quantification (LOQ) for PRG and MC were found to be 535 and 82 ng/band respectively.

**Robustness:** Acceptable %RSD values obtained after making small deliberate changes in the developed

HPTLC method indicate that the method is robust for the intended purpose (Table 4).

**Solution stability:** The solutions at analytical concentration PRG (50, 200, 300 ng/band) and MC (0.5, 10, 20 ng/band) were prepared and stored at room temperature for 24 h and analyzed at interval of 0, 6, 12 and 24 h for the presence of any band other than that of PRG and MC and the results were simultaneously compared with the freshly prepared PRG and MC standard solution of the same concentration in the form of change in %RSD of the response obtained. Standard solution of the same concentration in the form of change in %RSD of the response obtained.

## Method application

Marketed formulation was analyzed using proposed method which gave percentage recovery for PRG and MC more than 97%.

## Conclusions

This developed and validated method for simultaneous analysis of PRG and MC in pharmaceutical preparations is very rapid, accurate, and precise. The method was successfully applied for determination of PRG and MC in its pharmaceutical capsule formulation. Moreover it has advantages of short run time and the possibility of analysis of a large number of samples, both of which significantly reduce the analysis time per sample. Hence this method can be conveniently used for routine quality control analysis of PRG and MC in its pharmaceutical formulations.

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