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## ORIGINAL ARTICLE

# Validated inherent stability-indicating HPLC-DAD method for simultaneous determination of Pamabrom and Paracetamol in marketed formulation

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**ABSTRACT**

*A simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC with diode array detection method was developed and validated for the simultaneous determination of Pamabrom (PBM) and Paracetamol (PCM) in commercial tablets. PBM and PCM were degraded together under different stress test conditions prescribed by International Conference on Harmonization. The samples generated were used to develop a stability-indicating high performance liquid chromatographic (HPLC) method for both the drugs. The drugs were well separated from degradation products using a reversed-phase C 18 (4.6 x 250mm, 5µm particle size) column with isocratic elution of the mobile phase comprising of water: methanol: acetonitrile, in the ratio of 70:20:10 v/v/v. The mobile phase flow rate was maintained at 1.0 ml/min with the detection wavelength used for quantification of PBM and PCM was 279 nm. The drugs were subjected to different stress conditions like neutral, acidic and alkaline hydrolysis, oxidation, photolysis and thermal degradation. Degradation products produced was a result of stress studies did not interfered with the detection of PCB and PCM, thus the assay can thus be considered stability-indicating. Analytical performance of the proposed HPLC procedure was thoroughly validated with respect to system suitability, linearity, range, precision, accuracy, specificity, robustness, detection and quantification limits. The developed procedure is also applicable to the determination of instability of the drugs in commercial formulations.*

*Keywords: Pamabrom, Paracetamol, Stability indicating assay, forced degradation, HPLC, Diode array detection.*

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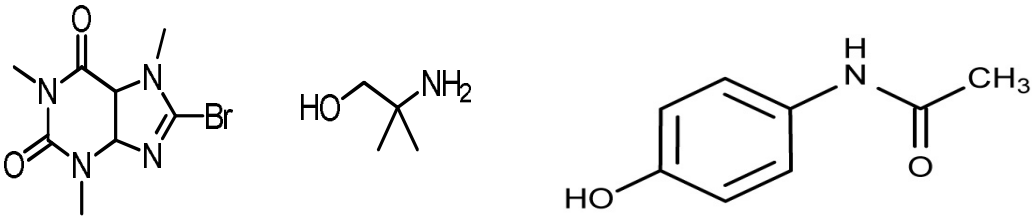
## INTRODUCTION

Pamabrom (PBM) is chemically, 1:1 mixture of 8-Bromo-3, 7-dihydro-1, 3-dimethyl-1H-purine-2, 6-dione with 2-amino-2-methyl-1-propanol (Fig.1a). It has a diuretic property [1-2]. It is official in US pharmacopoeia. It is assayed by liquid chromatography as per USP [3]. PBM, a xanthine derivative, is a safe and effective diuretic in relieving the water-accumulation symptoms of water-weight gain, bloating, swelling, and/or full feeling associated with the premenstrual and menstrual periods. It works, as all diuretics, by pulling excessive water from throughout the body. Paracetamol (PCM) chemically it is N-(4-hydroxyphenyl) acetamide (Fig. 1b). PCM is official in IP, BP, USP and JP [4-7]. It is classified as a mild analgesic and having antipyretic properties. It is much safer than aspirin in terms of gastric irritation, ulceration and bleeding.



a) b)

**Fig. 1: Chemical structure of a) Paracetamol b) Paracetamol**

In recent times, there is an increased tendency towards the development of stability-indicating assays, using the approach of stress testing as incorporated in the International Conference on Harmonization (ICH) guideline Q1AR(2). Even this approach is being extended to drug combinations, to allow accurate and precise quantitation of multiple drugs, their degradation products, and interaction products, if any.
The combination tablet containing PCM and PBM having NSAID and diuretic activity respectively and extensively used for the treatment of aches and pains due to muscle strain, spasm or overexertion as well as help to reduce periodic excess water retention for relief of pressure caused discomforts [9]. There are several HPLC procedures known for the analysis of PCM and PBM individually, and some methods even exist for simultaneous analysis of the two drugs, either in a combination with other drug or in biological fluids. Various UV, HPLC, HPTLC and stability indicating methods for PCM and PBM have been reported individually or in combination with other drugs [10-12]. To our knowledge, there is no RP-HPLC-DAD method reported for the combination available of an HPLC method with high sensitivity and selectivity will be very useful for the estimation of PCM and PBM in combined pharmaceutical dosage form in presence of their degradation product. Therefore the aim of study was to develop and validate sensitive, precise, accurate and specific RP-HPLC-DAD method for determination of PCM and PBM in formulation developed using the ICH approach of stress testing [13-16]. The method was also extended to marketed products.

## MATERIAL AND METHODS

### Materials

Working standards of pharmaceutical grade PCM, was received as gift sample from Emcure Pharmaceuticals Ltd, Pune and PBM was obtained as generous gift from Pan Drugs Ltd. Ahmadabad, India. Combination products containing the two drugs were purchased from local pharmacy shop. HPLC grade acetonitrile, water and methanol were purchased from Merck Chemicals, India.

### Equipment and chromatographic condition

The modular HPLC system used was equipped with Waters 510 HPLC pump with a Rheodyne injector (20 µl) and PDA 6000 LP detector. A Data Ace Chromatography Data system was used to record and evaluate the data collected during and following chromatographic analysis. The chromatographic separation was achieved on a Kromasil C 18, (250 mm × 4.6 mm i. d., and 5µm particle size). The eluent was monitored using photo diode array (PDA) detection at a wavelength of 279 nm. The mobile phase water: methanol: acetonitrile was used and column was maintained at ambient temperature at a flow rate of 1 ml/min. The mobile phase was filtered through a 0.45 µm nylon filter prior to use and degassed in an ultrasonic bath (Biomedica, India). A precision water bath equipped with MV controller (i-therm, Biomedica, India) was used to carry out selected reactions in solution. Thermal stability study was carried out in dry air oven (Biotechnics BTI–20D, Mumbai, India). Other equipments used were sonicator (Biomedica, India), analytical balance (Schimadzu AUX 220, Japan) and autopipettes (Eppendorf, Hamburg, Germany).

### Degradation studies

The study was intended to ensure the effective separation of PBM, PCM and its degradation peaks in the bulk drug and marketed formulation. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. In general, degradation studies were carried out at a concentration of 1 mg/ml of each drug and tablet formulation in the solution. All samples were then diluted in mobile phase to give a final concentration of 12.5 & 250µg/mL for PBM and PCM respectively and filtered before injection in the chromatographic system. For hydrolysis in water, the solution was refluxed for 2h at 70°C. Acid decomposition was carried out in 2M HCl and refluxed for 2h at 70°C and alkaline degradation was conducted using 2M NaOH and refluxed for 3.5h at 70°C. After cooling the solutions were neutralized and diluted with mobile phase. Degradation was also carried out in solid state by exposing pure drugs and formulation to dry heat at 70°C for 8 h, and in dark. The photochemical stability of the drug was studied by exposing the stock solution to intense UV radiation for 15 days.

Oxidation study was carried out using 3% oxidizing agent hydrogen peroxide for 9h at room temperature. Samples were withdrawn periodically and subjected to analysis after suitable dilution. The same stress conditions were applied to placebo and blank solution.

**Development of method**

HPLC studies were carried out on all the reaction solutions containing API individually, and on a marketed formulation. The separations were achieved by isocratic elution using water: methanol: acetonitrile (70:20:10 v/v/v) as a mobile phase. It was filtered through 0.45 µm nylon filter and degassed before use. The injection volume was 20µl and mobile phase flow rate was 1 ml/min. The detection was carried out at 279 nm.

**Preparation of tablets for assay**

Twenty tablets were weighed, crushed and mixed in a mortar and pestle for 20 min. A portion of powder equivalent to 125.0mg PCM and 6.25mg PBM was accurately weighed and transferred into each of six 50 ml A-grade volumetric flasks and sonicated for 20 min for complete dissolution of the PCB and PCM and the solutions were then diluted up to volume with mobile phase. Aliquots of the solution were filtered through a 0.45µm nylon filter and 1ml of the filtered solution was transferred to a 10ml A-grade volumetric flask and made up to volume with mobile phase, to yield final concentrations of drugs in the range of linearity previously described.

**Validation of the method**

The method was validated for linearity, precision (inter-day, intra-day), accuracy, specificity, selectivity, LOD, LOQ and robustness. Standard plots were constructed for both PBM and PCM in the range of 5-25 and 100-500 µg/ml. The experiment was repeated thrice on the same day and additionally on two consecutive days to determine intra- and inter-day precision, respectively. The method precision and system precision was determined by repeating the experiment six times. Accuracy was determined by fortifying the mixture of degraded solutions with three known concentrations of the drugs. Further, specificity of the method was assessed by study of the resolution factor of the drug peaks from nearest resolving peaks. The selectivity was determined by checking peak purity of all the peaks, including those of degradation products, using a PDA detector.

## RESULTS AND DISCUSSION

**Degradation behavior**

HPLC studies on the combination under different stress conditions indicated the following degradation behavior.

Acidic condition

Both the drugs were found to be highly labile showed degradation within 2h at 70°C in 2M HCl. PBM showed little higher degradation as compared to PCM. The major degradation products formed were at retention times (RTs) 0.5, 1.7 and 6.0 min.

Degradation in alkali

The combination showed sufficient degradation within 3.5h at 70°C in 2M NaOH. The major products appeared at RTs 2.4, 9.6 and 10.2 min.

Neutral (water) degradation

Sufficient degradation was observed upon refluxing the combination for 2h at 70°C. PBM showed little higher degradation as compared to PCM. The degradation products appeared at RTs 5.5 and 7.9 min.

Oxidative degradation

The drugs showed sufficient degradation when the combination was degraded in 3% $H_2O_2$ for 9h at room temperature. Similar to acid and alkaline degradation, PBM showed higher oxidative degradation. The two major degradation products appeared at 4.5and 7.4 min.

Thermal degradation

Dry heat degradation studies showed that the combination was unstable in dark. Enough degradation was observed when the combination was exposed to dry heat at 70 °C for 8h. PCM showed higher degradation than PBM. The major degradation products resolved at 0.7 and 11.1 min.

Photolytic degradation

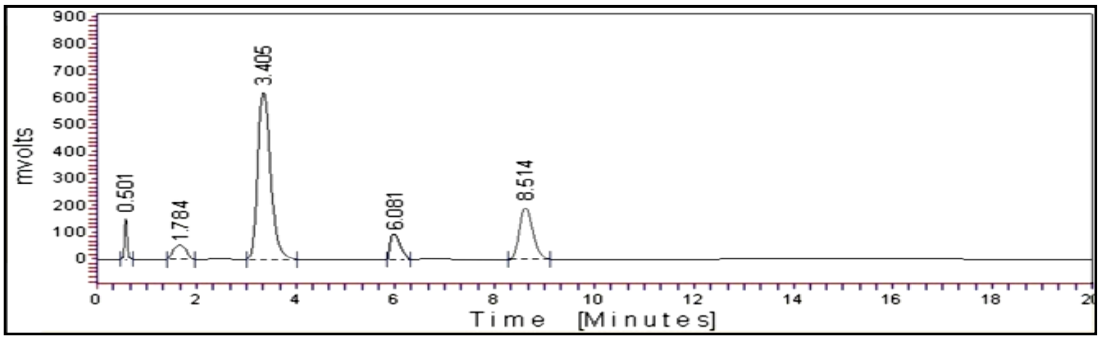
Drug was exposed to longer and shorter UV radiation 8h per day for 15 days, both drugs showed appreciable stability and did not influenced for photolytic degradation.

**Development and optimization of the stability-indicating HPLC method**

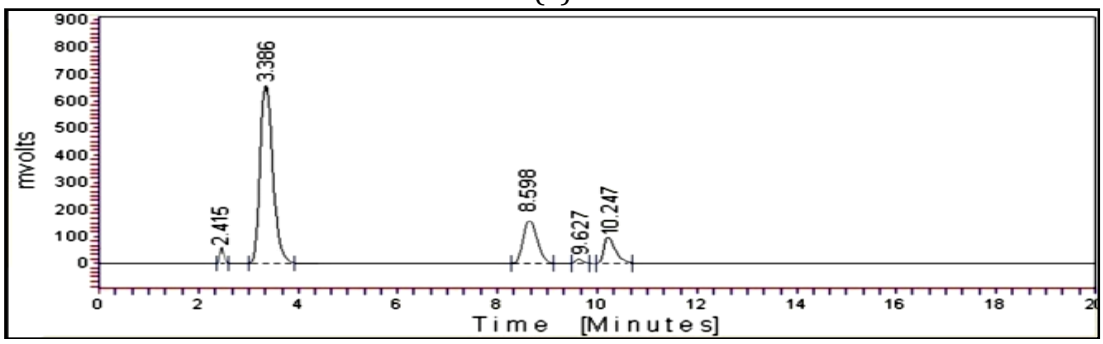
An isocratic method was found necessary to optimize the separation of major degradation products formed under various stress conditions. The best resolution was achieved with initial run of water: methanol: acetonitrile in the ratio of (70:20:10 v/v/v). The obtained chromatogram is represented in fig. 3 shows the Rt of PCM and PBM at 3.38 and 8.55 respectively. The method worked well with the mixture

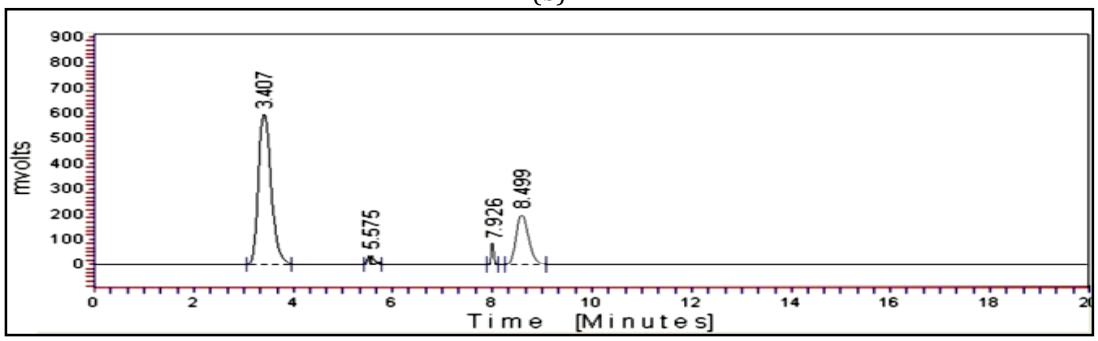
of degradation solutions and was even applicable to degraded formulations. Fig. 4(a–f) shows the obtained chromatographic resolution of PBM and PCM from its degradation product generated during various stress conditions.



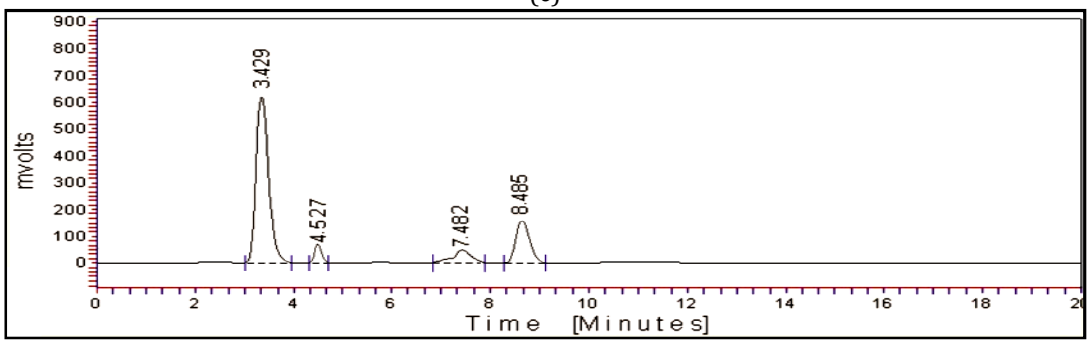

(a)




(b)

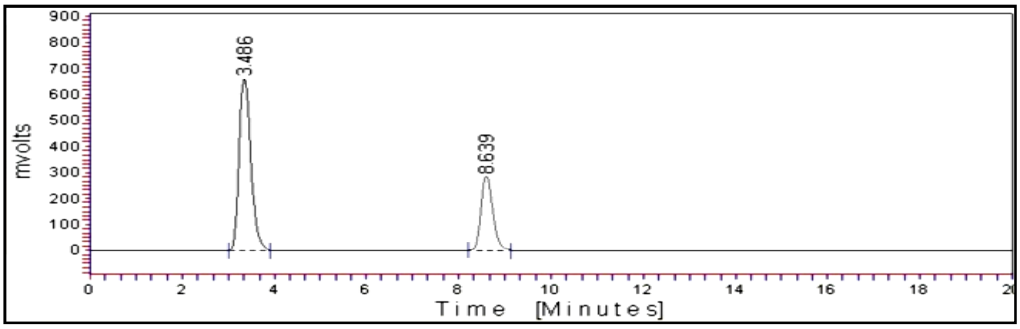



(c)

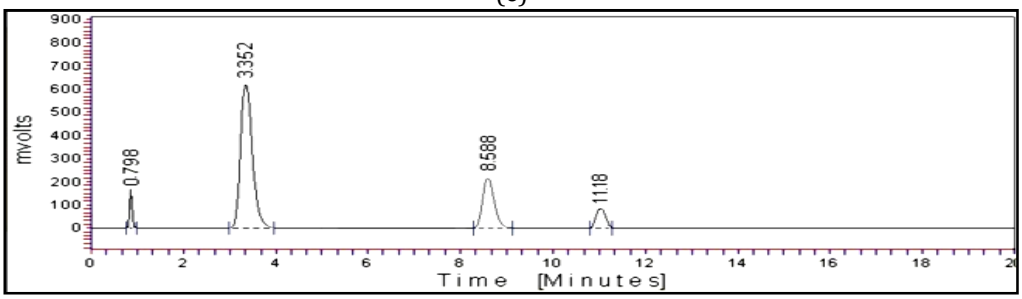



(d)




(e)




(f)

Fig.4. Indicates the obtained chromatograms of stress studies of PCM and PBM as : a) Acid degradation b) Alkali degradation c) Neutral degradation d) Oxidative degradation e) Photolytic degradation f) Thermal degradation

**Validation of the developed stability-indicating method**

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery and robustness/ruggedness.

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 5–25µg/ml and 100–500µg/ml for PBM ($n$ = 3) and PCM ($n$ = 3), respectively. Peak areas of PBM and PCM were plotted versus their respective concentrations and linear regression analysis performed on the resultant curves. Correlation coefficients ($n$=3) were found to be 0.999 for both the drugs with %RSD values ranging from 0.110 – 0.755 % across the concentration ranges studied. Typically, the regression equations were: $y$ = 20075$x$ + 38698 ($R$ = 0.999) for PCM and $y$ = 11839$x$ + 14459 ($R$ = 0.9993) for PBM, respectively.

LOQ and LOD

The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. The LOQ that produced the requisite precision and accuracy was found to be 12.99µg/ml for PCM and 0.95µg/ml for PBM, respectively. The LOD was determined based on signal-to-noise ratios and was determine using an analytical response of three times the background noise. The LOD for both PCM and PBM were found to be 4.28µg/ml and 0.23µg/ml, respectively.

Precision

The intra- and inter-day variability or precision data were summarized in table 1 and 2 respectively and were assessed by preparation of standard solutions to produce solutions of three different concentrations of PCM and PBM. Repeatability or intra-day precision was investigated by injecting six replicate samples of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the sample of three different concentrations over three consecutive days.

**Table: 1 Intra-day Precision Data**

| **Parameters** | **PCM** | | | **PBM** | | |
|---|---|---|---|---|---|---|
| | **Concentration (µg/ml )*** | | | **Concentration (µg/ml )*** | | |
| | **200** | **250** | **300** | **10** | **12.5** | **15** |
| **% Estimated** | 99.72 | 99.12 | 99.57 | 99.51 | 99.27 | 99.35 |
| **S. D.** | ± 0.6090 | ± 0.4050 | ± 0.4750 | ± 0.8280 | ± 0.4807 | ± 0.5901 |
| **C. V.** | 0.6107 | 0.4085 | 0.4771 | 0.8321 | 0.4842 | 0.5940 |

* Mean of six determinations, S.D: Standard Deviation, C.V: Coefficient of variance

**Table: 2 Inter-Day Precision Data**

| Parameters | PCM | | | PBM | | |
|---|---|---|---|---|---|---|
| | Concentration (µg/ml )* | | | Concentration (µg/ml )* | | |
| | 200 | 250 | 300 | 10 | 12.5 | 15 |
| % Estimated | 98.99 | 99.6 | 99.68 | 99.25 | 99.44 | 98.95 |
| S. D. | ± 0.3594 | ± 0.5682 | ± 0.7562 | ± 0.4932 | ± 0.6030 | ± 0.3881 |
| C. V. | 0.3631 | 0.5705 | 0.7586 | 0.4969 | 0.6064 | 0.3922 |

* Mean of six determinations, SD: Standard Deviation, C.V: Coefficient of variance

Accuracy

Accuracy study was performed by standard addition method by adding pure drug in a powder of marketed formulation at three different levels 80%, 100% and 120%. In each case, the percent relevant error and %RSD was calculated and found to be less than 0.41 for PCM and 0.83 PBM. The data obtained from recovery study for the determination of each compounds of interest are summarized in table 3.

**Table: 3 Statistical Validation for Recovery Study**

| Level of recovery | % Mean Recovery* | | Standard Deviation | | % R.S.D. | | S.E.M | |
|---|---|---|---|---|---|---|---|---|
| | PCM | PBM | PCM | PBM | PCM | PBM | PCM | PBM |
| 80 % | 98.86 | 99.53 | ± 0.268 | ± 0.832 | 0.271 | 0.835 | 0.155 | 0.480 |
| 100 % | 99.52 | 98.56 | ± 0.413 | ± 0.697 | 0.414 | 0.707 | 0.238 | 0.402 |
| 120 % | 99.44 | 98.75 | ± 0.232 | ± 0.276 | 0.233 | 0.279 | 0.134 | 0.159 |

*Average of three determinations, RSD: Relative Standard deviation, S.E.M: Standard error of mean

Specificity

The results of stress testing studies in addition to that of monitoring standard solutions of each drug in the presence of their degradants indicated a high degree of specificity of this method for both PCM and PBM. The degradation product(s) of each of the parent compounds was found to be similar for both the tablets and API powders assessed. The method has sufficient specificity and selectivity as the two drugs and even degradation products were well separated from each other, with the resolution factor of >2 in all cases. All the peaks were pure, which was proved through PDA purity studies. Data of peak purity index and purity threshold values indicates the degradants peaks are well separated from the drug peak. The established mass balance study ensured that all degradants were adequately detected. The above study is shown in table 4.

**Table : 4 Mass balance and peak purity study**

| Stress Condition | % degradation | | Purity Angle | | Purity Threshold | | % Assay* | | Mass Balance | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PCM | PBM | PCM | PBM | PCM | PBM | PCM | PBM | PCM | PBM |
| **Acid** 2 M HCl for 2h at 70°C. | 16.97 | 14.24 | 0.316 | 0.207 | 1.026 | 0.988 | 82.94 | 84.76 | 99.91 | 99 |
| **Alkaline** 2 M NaOH for 3.5h at 70°C | 5.84 | 13.25 | 0.297 | 0.213 | 0.997 | 0.891 | 92.61 | 85.7 | 98.45 | 98.95 |
| **Neutral** $H_2O$ for 2 h at 70°C | 3.0 | 07 | 0.197 | 0.288 | 0.953 | 0.926 | 96.2 | 91.8 | 99.2 | 98.8 |
| **Oxidative** 3% $H_2O_2$ for 9h at R.T | 9.10 | 12.86 | 0.243 | 0.338 | 0.855 | 0.935 | 90.02 | 86.2 | 99.12 | 99.06 |
| **Photolytic** 15 days UV-Vis radiation | 0.0 | 0.0 | 0.306 | 0.316 | 0.903 | 0.911 | 99.26 | 98.90 | 99.26 | 98.90 |
| **Thermal** 8h at 70°C | 9.61 | 5.9 | 0.419 | 0.253 | 0.968 | 1.005 | 89.53 | 93.5 | 99.14 | 99.4 |

*Average of three determinations

Robustness

The robustness of the method was investigated under a variety of conditions including changes of detection wavelength, flow rate and of organic phase composition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust as R.S.D was found to be <1%.

## CONCLUSION

This study presents a simple, rapid, accurate, precise, economic and validated stability-indicating HPLC method for simultaneous estimation of PCM and PBM in the presence of degradation products. The result

of stress testing undertaken according to the International Conference on Harmonization (ICH) guidelines reveals that the method is selective and stability-indicating. The method could be applied with success even to the analysis of marketed products, as no interference was observed due to excipients or other components present.

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**CONFLICT OF INTEREST**

We have no conflict of interest to declare.

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