5. Results and Discussion

In the present study, three novel RP-HPLC methods for the simultaneous determination of PHE, PCM & CPM (Method I), AMB, GPN & SBS (Method II) and PHE, PCM & CTH (Method III) in bulk drug and pharmaceutical formulations with UV detection were developed and validated as per ICH guidelines for analytical method validation, Q2 (R1).

5.1. Instrumentation and Chromatographic Conditions

A PerkinElmer HPLC system (USA) consisting of binary solvent delivery pump (series 200), vacuum degasser, rheodyne injector with a 20 μL loop, detector (series 200 UV/VIS) and Total Chrom Navigator software (version 6.3.2.0646) was used. Princeton sphere C-8, 25cm×4.6mm (Rankem) analytical and Merck C-18, 25cm×4.6mm columns, eluted with mobile phase at the flow rate of 1.0 mL.min⁻¹ were used.

5.2. Chemicals and Reagents

Standards were obtained from Sun Pharmaceutical (Mumbai, India) and Torrent Pharmaceutical, (Gandhinagar, India). Acetic acid, water, methanol and acetonitrile (HPLC-grade) were procured from Rankem, RFCL Limited, New Delhi, India. Ammonium acetate AR and di hydrogen potassium phosphate AR grade were procured from Central Drug House (P) Limited, New Delhi, India. The 0.45-μm pump nylon filter was obtained from Advanced Micro devices (Ambala Cantt, India). Other chemicals used were of analytical or HPLC grade.

5.3. Identification of Drugs

The identity of selected drugs was ascertained from certificate of analysis and was further established by initial observations (Physical appearance/ characteristics), melting point determination, thin layer chromatography, IR spectroscopy and UV spectroscopy. The obtained values complied with the official specifications.

5.4. Method Development

The chromatographic methods were developed and validated by assessing linearity, accuracy, selectivity, precision and robustness. The focus was on the selection of mobile phase and its composition as well as other parameters like flow rate and working wave length. Several mobile
phase compositions were tried to resolve the peaks. One major problem with the analysis of basic drugs is peak tailing since they strongly interact with polar ends of HPLC column packing materials causing severe peak asymmetry and low separation efficiencies. During the optimization of the method, two columns (Princeton sphere C-8, 25cm×4.6mm and Merck C-18, 15cm×4.6mm) and two organic solvents (acetonitrile and methanol) were used. The chromatographic conditions were also optimized by using different buffers in mobile phase preparation. After a series of screening experiments, it was noted that phosphate buffer (0.02M & 0.01M potassium dihydrogen phosphate) gave better peak shapes than their acetate counterparts. On the other hand, pH was adjusted between 7.0 and 3.0. (pH 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5 and 3.0).

Finally, the mobile phase consisting of phosphate buffer (0.02M potassium dihydrogen phosphate) and acetonitrile in the ratio of 65: 35 v/v (pH 4.2 ± 0.01, adjusted with 50% ortho phosphoric acid) was selected for Method I (PHE, PCM & CPM) because it resolved the peaks of PHE ($t_a = 2.32±0.02$ min), PCM ($t_a = 3.24±0.03$ min) and CPM ($t_a = 9.84±0.02$ min) efficiently. Quantification was done on the basis of peak area at 1.0 mL.min$^{-1}$ flow rate with UV detection at 235 nm at room temperature.

The mobile phase encompassing phosphate buffer (0.01M potassium dihydrogen phosphate), acetonitrile and tri ethyl amine in the ratio of 48: 52: 0.5 v/v (pH 5.6 ± 0.01, adjusted with 50% ortho phosphoric acid) was selected for Method II (AMB, GPN & SBS) because it resolved the peaks of AMB ($t_a = 2.74±0.03$ min), GPN ($t_a = 4.30±0.02$ min) and SBS ($t_a = 6.09±0.02$ min) efficiently. Quantification was done on the basis of peak area at 1.0 mL.min$^{-1}$ flow rate with UV detection at 215 nm at room temperature.

The mobile phase comprising of phosphate buffer (0.02M potassium dihydrogen phosphate), acetonitrile in the ratio of 53: 47 v/v (pH 4.8 ± 0.01, adjusted with 50% ortho phosphoric acid) was selected for Method III (PHE, PCM & CTH) because it resolved the peaks of PHE ($t_a = 2.44±0.03$ min), PCM ($t_a = 3.42±0.02$ min) and CTH ($t_a = 7.60±0.02$ min) efficiently. Quantification was done on the basis of peak area at 1.0 mL.min$^{-1}$ flow rate with UV detection at 242 nm at room temperature.
5.5. Method Validation

After method development, the RP-HPLC methods were validated with respect to parameters such as specificity, linearity, range, stability, accuracy, precision, LOD and LOQ as per internationally accepted ICH guidelines.

5.5.1. Specificity

Specificity of the method was established by comparing chromatograms of each drug with the chromatograms of mixture of drugs. The specificity study revealed no interference by the excipients, since none of the peaks appeared at the retention time of selected drugs. Selectivity of the developed methods was confirmed by the fact that the resolution between the peaks found to be more than 2.0. This suggests that under the proposed chromatographic conditions all drugs were completely and effectively separated from each other.

5.5.2. Range

The range of analytical method is the interval between the upper and lower levels of the analytes. Linearity was observed in the concentration range of 2-14 µg.mL\(^{-1}\), 6-42 µg.mL\(^{-1}\), and 5-35 µg.mL\(^{-1}\) for Method I (PHE, PCM & CPM), Method II (AMB, GPN & SBS) and Method III (PHE, PCM & CTH) respectively.

5.5.3. Linearity

The responses were measured as peak area. The calibration curves obtained by plotting peak area (in mV.s) against concentration (µg/mL) showed linearity. The best fit lines for the calibration curves were obtained by a linear regression equation.

For Method I, regression lines were found to be \(y = 223270x + 13400\), \(y = 217913x + 34828\) and \(y = 224208x + 21400\) for PEH, PCM and CPM, respectively. Correlation coefficient values for PHE, PCM and CPM were noted to be 0.9998, 0.9996 and 0.9998, respectively. This indicated high degree of linearity for all the three drugs.

For Method II, regression lines were found to be \(y = 237831x - 64315\), \(y = 236641x + 21400\) and \(y = 231075x + 95828\) for AMB, GPN and SBS, respectively. Correlation coefficient values for
AMB, GPN and SBS were noted to be 0.9997, 0.9999 and 0.9997, respectively. This indicated high degree of linearity for all the three drugs.

For Method III, regression lines were found to be $y = 185112x + 54257$, $y = 192112x - 14315$ and $y = 31397x + 5685.3$ for PHE, PCM and CTH, respectively. Correlation coefficient values for PHE, PCM and CTH were noted to be 0.9999, 0.9998 and 0.9997, respectively. This indicated high degree of linearity for all the three drugs.

5.5.4. Precision
The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of the methods was assessed by inter day and intraday analysis. The low value of % RSD showed that the method is precise within the acceptance limit of ±2%. The results indicated good precision of the developed methods.

5.5.5. Accuracy
The recovery experiments were performed by adding known amounts of the drugs in the placebo at three levels: 80%, 100%, and 120% of the label claim of the marketed formulation. Three samples were prepared for each recovery level. The solutions were then analyzed, and percentage recoveries were calculated from the calibration curves. The mean recovery values for PHE, PCM and CPM (Method I) were found to be 99.87%, 100.62% and 100.16%, respectively. The mean recovery values for AMB, GPN and SBS (Method II) were noted to be 100.27%, 101.08% and 100.86%, respectively. The mean recovery values for PHE, PCM and CTH (Method III) were found to be 100.36%, 100.31% and 100.01%, respectively. The results revealed that there was no interference of excipients.

5.5.6. The Limit of Detection and Limit of Quantitation
The LOD and LOQ predict the sensitivity of the method. The approach based on the standard deviation (SD) of response and slope (m) was used for determining the detection and quantitation limits.
The LOD values for Method I, PHE, PCM and CPM were noted to be 0.28, 0.36 & 0.52 μg.mL\(^{-1}\), respectively while LOQ values were noted to be 0.86, 1.1 & 1.4 μg.mL\(^{-1}\), respectively. The values indicated that the method is sensitive.

The LOD values for Method II, AMB, GPN and SBS were found to be 0.18, 0.12 & 0.28 μg.mL\(^{-1}\), respectively while LOQ values were found to be 0.49, 0.37 & 0.68 μg.mL\(^{-1}\), respectively. The values indicated that the method is sensitive.

The LOD values for Method III, PHE, PCM and CTH were noted to be 0.52, 0.39 & 0.19 μg.mL\(^{-1}\), respectively while LOQ values were noted to be 1.47, 0.85 & 0.51 μg.mL\(^{-1}\), respectively. The values indicated that the method is sensitive.

### 5.5.7. Analysis of Marketed Formulation

The developed methods were successfully applied to analyze PHE, PCM & CPM (Method I), AMB, GPN & SBS (Method II) and PHE, PCM & CTH (Method III) in marketed tablet formulations. The amounts recovered were expressed as percent of the label claim. Analyses of marketed tablets, Maxtra P Tab (Batch no. M2431, Zuventus Healthcare Ltd.), Respira Tab (Batch no. RT650, Geno Pharmaceuticals Ltd), Allercet-DC Tab, (Batch no. Adad1976, Micro labs Ltd) were carried out using optimized mobile phases and HPLC conditions. The recovery values of analysis of tablets obtained by the proposed methods were found between 99.64 & 100.58%, 98.06 & 101.5% and 99.2 & 101.24%, for Method I (PHE, PCM & CPM), Method II (AMB, GPN & SBS) and Method III (PHE, PCM & CTH) respectively. This showed that the estimation of dosage forms complied to the acceptance level of 98% to 102%.

### 5.6. Forced Degradation Studies

The forced degradation studies were conducted to evaluate the stability-indicating capability and selectivity of the developed methods.

#### 5.6.1. Acid Degradation

The chromatogram showed major degradation products at retention times of 2.16 min, 7.70 min with the standard peak at 2.37 min, 3.45 min and 9.91 min for Method I (PHE, PCM & CPM).

The chromatogram showed major degradation products at retention times of 1.92 min, 8.12 min, 9.27 min, 11.75 min with the standard peak at 2.74 min, 4.35 min and 6.91 min for Method II (AMB, GPN & SBS).
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The chromatogram showed major degradation products at retention times of 2.22 min, 2.31 min, 5.31 min, 9.88 min with the standard peak at 2.47 min, 3.45 min and 7.40 min for Method III (PHE, PCM & CTH).

5.6.2. Base Degradation

The chromatogram showed major degradation products at retention times of 2.10 min, 3.21 min, 11.34 min with the standard peak at 2.33 min, 3.19 min and 5.84 min for Method I (PHE, PCM & CPM).

The chromatogram showed major degradation products at retention times of 1.12 min, 3.47 min, 9.11 min with the standard peak at 2.52 min, 4.90 min and 7.14 min for Method II (AMB, GPN & SBS).

The chromatogram showed major degradation products at retention times of 2.16 min, 5.31 min, 7.70 min, 9.88 min, 27.56 min with the standard peak at 2.47 min, 3.45 min and 7.40 min for Method III (PHE, PCM & CTH).

5.6.3. Oxidative Degradation

The chromatogram showed major degradation products at retention times of 2.01 min, 11.19 min with the standard peak at 2.33 min, 3.19 min and 5.72 min for Method I (PHE, PCM & CPM).

The chromatogram showed major degradation products at retention times of 1.21 min, 5.14 min, 10.34 min with the standard peak at 2.21 min, 3.68 min and 7.95 min for Method II (AMB, GPN & SBS).

The chromatogram showed major degradation products at retention times of 2.34 min, 6.92 min, 27.79 min with the standard peak at 2.46 min, 3.43 min and 7.43 min for Method III (PHE, PCM & CTH).

5.6.4. Thermal Degradation

The chromatogram showed major degradation products at retention times of 1.01 min, 5.86 min, 7.32 min with the standard peak at 2.49 min, 3.55 min and 9.88 min for Method I (PHE, PCM & CPM).
The chromatogram showed major degradation products at retention times of 1.52 min, 2.01 min, 9.32 min with the standard peak at 2.76 min, 3.85 min and 8.35 min for Method II (AMB, GPN & SBS).
The chromatogram showed major degradation products at retention times of 2.52 min, 4.34 min with the standard peak at 2.23 min, 3.01 min and 6.78 min for Method III (PHE, PCM & CTH).

5.6.5. Photo Degradation
The chromatogram showed major degradation products at retention times of 1.9 min, 2.02 min, 2.21 min, with the standard peak at 2.43 min, 3.45 min and 9.26 min for Method I (PHE, PCM & CPM).
The chromatogram showed major degradation products at retention times of 1.67 min, 4.72 min with the standard peak at 2.35 min, 4.11 min and 8.60 min for Method II (AMB, GPN & SBS).
The chromatogram showed major degradation products at retention times of 2.34 min, 6.92 min, 7.79 min with the standard peak at 2.46 min, 3.43 min and 7.43 min for Method III (PHE, PCM & CTH).