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Research Article

Application of HPLC for the Simultaneous Determination of Paracetamol, Chlorzoxazone, and Nimesulide in Pharmaceutical Dosage Form

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A simple, precise, and accurate reversed-phase liquid chromatographic method has been developed for the simultaneous determination of paracetamol (PCM), chlorzoxazone (CHZ), and nimesulide (NIM) in pharmaceutical dosage form. The chromatographic separation was achieved on a Thermo Hypersil GOLD C_{18} column (250 × 4.6 mm i.d., 5 μm particle size). The mobile phase consisted of water : acetonitrile (55 : 45 v/v). The flow rate was set to 1.2 mL min^{-1} and UV detection was carried out at 275 nm. The retention time (t_R) for PCM, CHZ, and NIM was found to be 2.69 ± 0.02, 4.61 ± 0.01, and 9.55 ± 0.02 min, respectively. The validation of the proposed method was carried out for linearity, precision, robustness, limit of detection, limit of quantitation, specificity, and accuracy. The linear dynamic ranges were 32.5–65.0 μg mL^{-1} for PCM, 37.5–75.0 μg mL^{-1} for CHZ, and 10.0–20.0 μg mL^{-1} for NIM. The developed method can be used for routine quality control analysis of titled drugs in pharmaceutical dosage form.

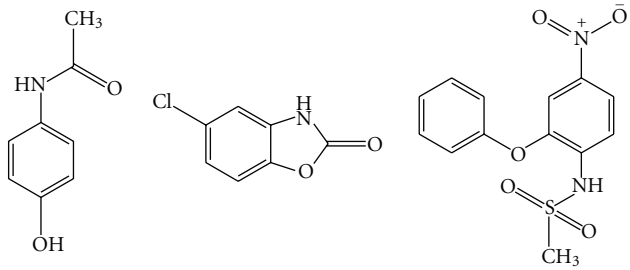
1. Introduction

Multiple component formulations have been taking up the market nowadays due to their synergistic effects, quick relief, multiple actions, tolerability, and patient acceptance. Many such combinations are available in market, one of such formulations is combination of Paracetamol (PCM), Chlorzoxazone (CHZ), and Nimesulide (NIM).

Paracetamol (PCM) (Figure 1), (N-(4-hydroxyphenyl) acetamide, is a para-aminophenol derivative and nonopiate, nonsalicylate, centrally and peripherally acting analgesic agent. It has weak anti-inflammatory effects. The most commonly consumed daily dose, 1000 mg, results in roughly 50% inhibition of both COX-1 and COX-2 in whole blood assays *ex vivo* in healthy volunteers. It has been suggested that COX inhibition might be disproportionately pronounced in the brain, explaining its antipyretic efficacy and its direct activity on the centre for the body temperature regulation in the hypothalamus. It is official in Indian Pharmacopoeia (IP) and British Pharmacopoeia (BP). Both IP and BP suggest titrimetric and UV spectrophotometric assay method for paracetamol in bulk and tablet formulations [1–3].

Chlorzoxazone (CHZ) (Figure 1), (5-chloro-2(3H)-benzoxazolone), inhibits histamine release and has skeletal muscle relaxant property. It is used to decrease muscle tone and tension and thus to relieve spasm and pain associated with musculoskeletal disorders. It is official in United States Pharmacopeia (USP) [4, 5].

Nimesulide (NIM) (Figure 1), N-(4-nitro-2-phenoxyphenyl) methanesulfonamide, is a derivative of p-nitrophenylmethanesulfonamide. It belongs to selective COX-2 inhibitors, with a potent anti-inflammatory and analgesic activity, when administered orally, rectally, or topically. Due to its analgesic and antipyretic properties, it is widely used for the treatment of various inflammatory processes. It is



PCM CHZ NIM

Figure 1

approved for use in treatment of musculoskeletal disorder, thrombophlebitis, dental pain, and inflammation [6–8].

All the three drugs are coformulated as a pharmaceutical dosage form for relief of pain associated with musculoskeletal disorder.

The literature survey reveals that PCM is estimated individually or in combination with other drugs by UV [9–12], HPLC [13–22], and in plasma [23, 24] by RP-HPLC. In addition, LC-MS [25, 26], HPTLC [27–33], and stability-indicating GC/MS method [34] have been reported.

For CHZ, UV method [15, 16, 18, 35–37] RP-HPLC method [38, 39], HPLC/UV method for analysis of plasma/serum samples [40, 41] and HPTLC [33] methods have been reported.

Similarly, for NIM UV method [37, 42, 43], RP-HPLC [38, 44–47] and HPTLC [33, 48] methods have been reported.

Reported liquid chromatographic methods [49] for detection of 18 NSAIDs are time consuming, expensive, cumbersome, and tedious. The methods used gradient mobile phase with different pH aqueous solvents, varying column temperature and organic solvent concentration (methanol) which is tedious for the analysis of formulations containing only these three drugs (PCM, CHZ, and NIM). The authors in the present paper proposed a validated quantitative HPLC-UV method for the simultaneous analysis of three drugs (PCM, CHZ, and NIM) in their combined pharmaceutical dosage form which is a simple isocratic method that consists of water : acetonitrile (55 : 45 v/v) as mobile phase with UV detection at a fixed wavelength of 275 nm thus provides a greater sensitivity for the quantitation of the these drugs. Moreover, UPLC system is costly and such sophisticated instrument may not be readily available in many organizations. Therefore, HPLC method described in the present paper thus can be used for the routine analysis in those laboratories that lack UPLC systems.

Hence, it was found desirable to develop a specific and precise LC method, which can be applied in the product development laboratories and also in the quality control laboratories to ensure the quality of the product. This paper describes the development, validation, and application of the proposed method for the simultaneous estimation of PCM, CHZ, and NIM in pharmaceutical dosage form.

2. Experimental

2.1. Chemicals and Reagents. Working standards of pharmaceutical grade PCM (99.6%, w/w), and CHZ (99.2%, w/w), NIM (99.8%, w/w) were obtained as gift sample from Cipla Pharmaceuticals, Maharashtra, India. Fixed dose combination Tablet (NICIP MR, batch no. AH0313, Exp. Date: 11/13, Mfg. Date: 12/10, Cipla Pharmaceuticals Ltd.) containing 325 mg of PCM, 375 mg of CHZ, and 100 mg of NIM was purchased from local pharmacy, Pune, India. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India. High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

2.2. Instrumentation and Chromatographic Conditions. The HPLC system (Jasco corporation, Tokyo, Japan) consisted of a Pump (model Jasco PU-2080 Plus) along with manual injector sampler programmed at 20 μL capacity per injection that was used. The UV/VIS model Jasco UV 2075 detector was used. LC separations were performed on a Thermo Hypersil GOLD C_{18} column (250 $\times$ 4.6 mm i.d., 5 μm particle size). Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. The mobile phase consisted of water : acetonitrile (55 : 45 v/v). The flow rate was set to 1.2 mL min^{-1} and UV detection was carried out at 275 nm.

2.3. Selection of Analytical Wavelength. Stock solutions of drugs were prepared in methanol separately. UV spectrum of 10 μg mL^{-1} of each individual drug was taken.

2.4. Solution Preparation. Stock standard solution containing PCM (3250 μg mL^{-1}), CHZ (3750 μg mL^{-1}), and NIM (1000 μg mL^{-1}) was prepared by dissolving 325 mg of PCM, 375 mg of CHZ, and 100 mg of NIM in 100 mL methanol. This was further diluted with water : acetonitrile 50 : 50 (v/v) (denoted "diluent") to obtain working standard solutions in a concentration range of 32.5–65.0 μg mL^{-1} (i.e., 32.5, 39.0, 45.5, 52.0, 58.5, and 65.0 μg mL^{-1}) for PCM, 37.5–75.0 μg mL^{-1} (i.e., 37.5, 45.0, 52.5, 60.0, 67.5, and 75.0 μg mL^{-1}) for CHZ, and 10.0–20.0 μg mL^{-1} (10.0, 12.0, 14.0, 16.0, 18.0, and 20.0 μg mL^{-1}) for NIM.

2.5. Sample Preparation. To determine the content of PCM, CHZ and NIM simultaneously in pharmaceutical dosage form NICIP-MR (label claim: 325 mg PCM, 375 mg CHZ, and 100 mg NIM per tablet, B. No. AH0313, Cipla Pharmaceuticals Ltd.), twenty tablets were weighed and finely powdered. An accurate weight of the powder equivalent to 325 mg of PCM, 375 mg of CHZ, and 100 mg of NIM was weighed. This was then transferred into a 100 mL volumetric flask containing 80 mL methanol, sonicated for 30 min with intermittent shaking, and diluted to 100 mL with methanol. This solution was filtered through a 0.45 μm nylon syringe filter. 0.7 mL of the above solution was transferred to 50 mL volumetric flask and diluted to volume with diluent. The concentration achieved after the above dilution was 45.5 μg mL^{-1} for PCM, 52.5 μg mL^{-1} for CHZ,

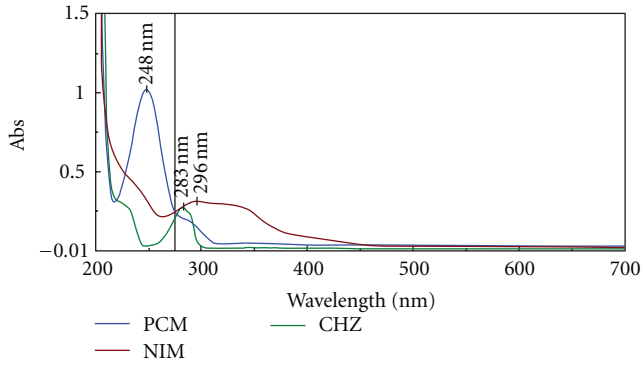


Figure 2: UV absorption spectra of PCM, CHZ, and NIM.

and 14.0 μg mL^{-1} for NIM. A constant 20 μL volume of sample solution was injected six times under the conditions described above. The peak areas were measured at 275 nm for PCM, CHZ, and NIM, respectively, and their concentrations in the samples were determined using multilevel calibration curve developed on the same HPLC system under the same conditions using linear regression equation.

2.6. Method Validation. The optimized HPLC method was validated with respect to the following parameters. The validation was performed as per the ICH guidelines [50].

2.6.1. Linearity. Constant volume of 20 μL injections were made for each concentration six times and chromatographed under the above-mentioned conditions. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. Linear calibration curves were generated using least-squares linear-regression analysis. Residual analysis was performed to ascertain linearity.

2.6.2. Precision. Precision of the method was determined with the standard and the real sample. The intraday and interday variation, for determination of PCM, CHZ, and NIM were carried out at three different standard concentration levels of 32.5, 45.5, and 65.0 μg mL^{-1} for PCM, 37.5, 52.5, and 75.0 μg mL^{-1} for CHZ, and 10.0, 14.0, and 20.0 μg mL^{-1} for NIM. Method repeatability was achieved by repeating the same procedure six times on the same day for intraday precision. The intermediate (interday) precision of the method was checked by performing same procedure on different days under the same experimental conditions. The repeatability of sample application and measurement of peak area were expressed in terms of relative standard deviation (% RSD) and standard error (SE). An amount of the sample powder equivalent to 100% of the label claim of PCM, CHZ and NIM was accurately weighed and assayed. System repeatability was determined by six replicate applications and measurement of real sample solution at 100% of the test concentration at 45.5 μg mL^{-1} for PCM, 52.5 μg mL^{-1} for CHZ, and 14.0 μg mL^{-1} for NIM, and the peak areas for real sample were expressed in terms of relative standard deviation (% RSD) and standard error (SE).

2.6.3. Robustness. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The robustness of the method was studied by deliberately varying parameters like flow rate ($\pm$0.1 mL min^{-1}), mobile phase composition ($\pm$1%), and two analytical columns, one (Hypersil GOLD C_{18} column) from Thermo Scientific, USA, and the other (HiQ-Sil HS C_{18} column) from Kromatek, Japan, were used during the experiment. Robustness of the method was done at three different concentrations 32.5, 45.5, and 65.0 μg mL^{-1} for PCM, 37.5, 52.5, and 75.0 μg mL^{-1} for CHZ, and 10.0, 14.0, and 20.0 μg mL^{-1} for NIM.

2.6.4. Limit of Detection and Limit of Quantitation. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ of PCM, CHZ, and NIM were determined by calibration curve method. LOD and LOQ were calculated by using following:

$$\text{LOD} = \frac{3.3 \times S_{y \cdot x}}{\text{S}} \qquad \text{LOQ} = \frac{10.0 \times S_{y \cdot x}}{\text{S}}, \tag{1}$$

where $S_{y \cdot x}$ is standard deviation of residuals from line; S is slope.

2.6.5. Specificity. The ability of an analytical method to unequivocally assess the analyte in the presence of other components. The specificity of the method was determined by the complete separation of PCM, CHZ and NIM along with other parameters like retention time (t_R), capacity factor (k), tailing or asymmetrical factor (T), and so forth. In addition, the specificity was analyzed in the real sample for separation from excipients which are generally present.

2.6.6. System Suitability. The system suitability parameters with respect to theoretical plates (N), peak symmetry (T), capacity factor (K'), selectivity (α), HETP (H), and resolution (Rs) between PCM, CHZ, and NIM peaks were defined.

2.6.7. Accuracy. Accuracy of the method was carried out by applying the method to drug sample to which known amounts of PCM, CHZ, and NIM standard powder corresponding to 80, 100, and 120% of label claim had been added (standard addition method). At each level of the amount six determinations were performed and the results obtained were compared with expected results.

2.6.8. Solution Stability. To check the stability of the drug by use of proposed method, freshly prepared solution of the analytes was injected into the system after intervals of 3 h, 24 h, and 48 h. Decomposition of drug was not observed during chromatogram development and no change in the peak area of the drug was observed during stability studies.

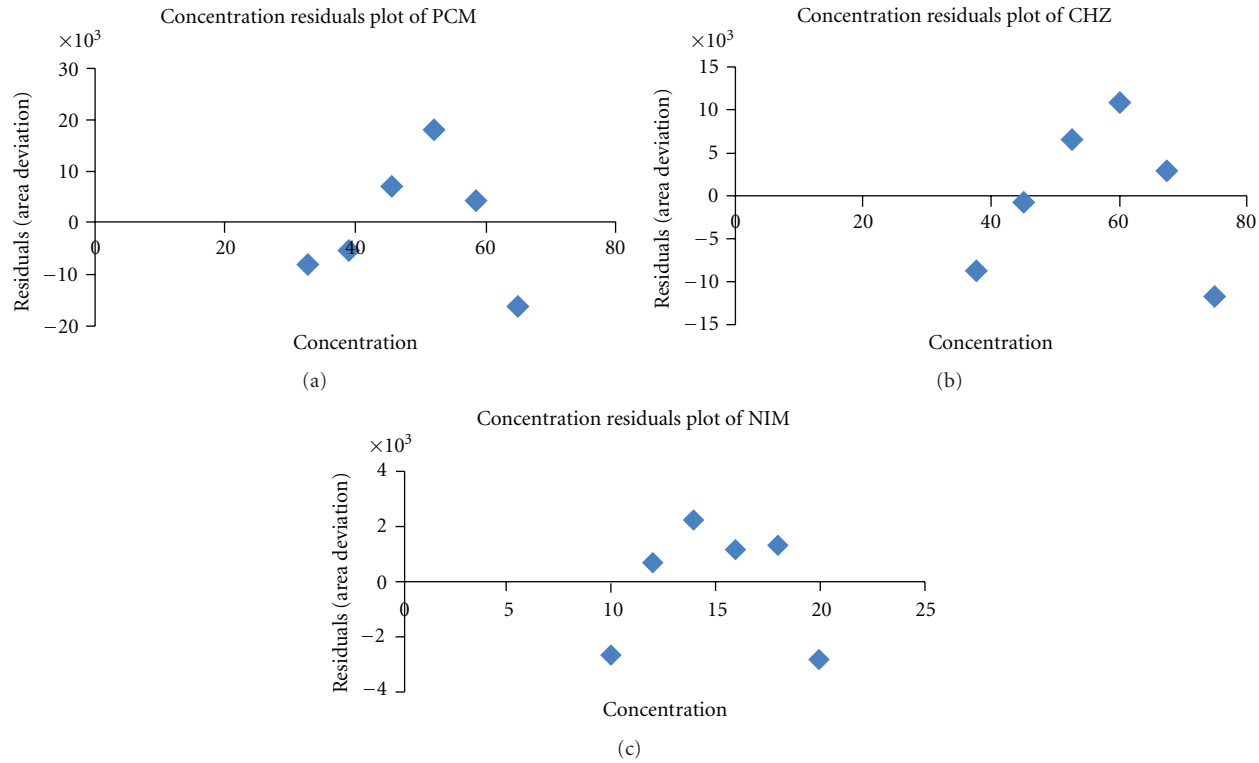


Figure 3: Concentration Versus Residual plot of PCM, CHZ, and NIM.

3. Results and Discussion

3.1. Selection of Analytical Wavelength. PCM, CHZ, and NIM showed maximum absorbance at 248 nm, 283 nm, and 296 nm, respectively. 275 nm was selected as a detection wavelength as the absorbance shown by all the three components was at the detectable range (Figure 2).

3.2. Optimization of HPLC Method. The HPLC procedure was optimized with a view to develop a simultaneous assay method for PCM, CHZ, and NIM, respectively. The stock standard solution was diluted with diluent to a concentration of 45.5 μg mL^{-1} for PCM, 52.5 μg mL^{-1} for CHZ, and 14.0 μg mL^{-1} for NIM. Then, the standard solution was injected into a Thermo Hypersil GOLD C_{18} column (250 × 4.6 mm i.d., 5 μm particle size). Initially, water : acetonitrile in 50 : 50 ratio was tried. Peaks of PCM and CHZ coeluted and also good asymmetric well-resolved peak of NIM was obtained. The problem of co-elution of PCM and CHZ peaks was resolved by reduction of the acetonitrile content in the mobile phase. Hence, the ratio of mobile phase was then changed to water : acetonitrile (55 : 45), which resulted in complete separation of all three compounds with t_R of 3.2 for PCM, 5.0 for CHZ, and 11.9 for NIM. However, to reduce the run time of analysis it was decided to increase the flow rate to 1.2 mL min^{-1} which also led to improved peak shape of all the three drugs. The optimum mobile phase was then found to be water : acetonitrile (55 : 45) with flow rate of 1.2 mL min^{-1} and UV detection was carried out at 275 nm which resulted in elution of all three compounds at t_R of 2.701 for PCM, 4.608 for CHZ, and 9.511 for NIM with acceptable plates, asymmetry, and good resolution.

3.3. Linearity. Linear relationships were observed by plotting drug concentration against peak areas for each compound. PCM, CHZ, and NIM showed linear response in the concentration range of 32.5–65.0 μg mL^{-1}, 37.5–75.0 μg mL^{-1} and 10.0–20.0 μg mL^{-1}, respectively. The corresponding linear regression equation was $y = 37777x - 51075$, $y = 19922x - 33423$, and $y = 23690x - 10637$, with square of correlation coefficient (r^2) of 0.9993, 0.9990, and 0.9994 for PCM, CHZ, and NIM, respectively. The linearity of calibration graphs and adherence of the system to Beer's law were validated by high value of correlation coefficient. No significant difference was observed in the slopes of standard curves. (Table 1). Residual analysis was performed to ascertain linearity (Figure 3).

3.4. Precision. The % RSD values depicted in Table 2 shows that proposed method provides acceptable intra-day and inter-day variation for PCM, CHZ, and NIM with respect to working standard.

The repeatability of real sample application and measurement of peak areas were expressed in terms of % RSD and were found to be 0.65 for PCM, 0.83 for CHZ and 0.95 for NIM.

3.5. Robustness. Each factor selected (except columns from different manufacturers) to examine was charged at three

Table 1: Linear regression data for calibration curves ($n = 6$).

Parameters	PCM	CHZ	NIM
Linearity range (μg mL^{-1})	32.5–65.0	37.5–75.0	10.0–20.0
Slope ± standard error	37777 ± 509.8	19922 ± 308.7	23690 ± 292.3
Intercept ± standard error	−51075 ± 25490	−33423 ± 17810	−10637 ± 4497
Confidence limit of slope[a]	36360 to 39190	19060 to 20780	22880 to 24500
Confidence limit of intercept[a]	−58150 to −44000	−38370 to −28480	−11890 to −9389
r^2	0.9993	0.9990	0.9994
$S_{y \cdot x}$[b]	13860	9687	2446

[a]95% confidence intervals.
[b]Standard deviation of residuals from line.

Table 2: Intraday and interday precision of PCM, CHZ, and NIM ($n = 6$).

Drugs	Concentration (μg mL^{-1})	Repeatability			Intermediate precision		
		found conc. ± SD	% R.S.D.	S.E.	found conc. ± SD	% R.S.D.	S.E.
PCM	32.5	32.65 ± 0.36	1.10	0.15	32.55 ± 0.20	0.61	0.09
	45.5	45.65 ± 0.25	0.55	0.10	45.75 ± 0.45	0.98	0.18
	65.0	65.65 ± 0.55	0.88	0.22	65.20 ± 0.35	0.54	0.14
CHZ	37.5	37.75 ± 0.36	0.95	0.14	45.56 ± 0.45	0.98	0.18
	52.5	52.60 ± 0.20	0.38	0.09	52.75 ± 0.46	0.87	0.19
	75.0	75.50 ± 0.70	0.93	0.29	75.15 ± 0.99	0.86	0.40
NIM	10.0	10.15 ± 0.10	0.99	0.04	10.12 ± 0.17	1.66	0.07
	14.0	14.05 ± 0.18	1.28	0.07	14.97 ± 0.25	1.67	0.10
	20.0	20.08 ± 0.15	0.75	0.06	20.28 ± 0.20	0.98	0.09

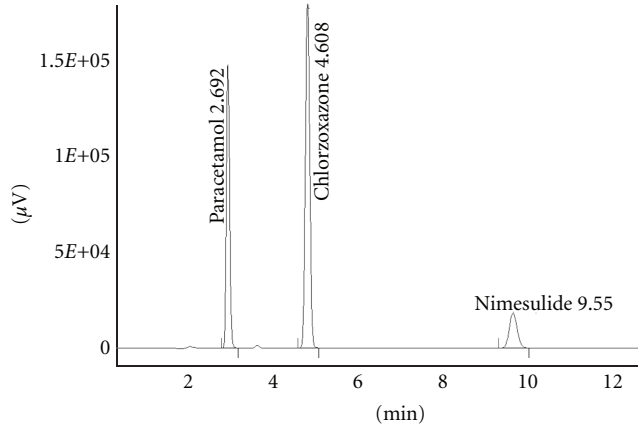


Figure 4: Chromatogram of pharmaceutical dosage form containing 45.5 μg mL^{-1} of PCM (t_R = 2.692), 52.5 μg mL^{-1} of CHZ, (t_R = 4.608) and 14.0 μg mL^{-1} of NIM (t_R = 9.550).

levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections ($n = 6$) of mixed standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Results, presented in Table 3 indicate that the selected factors remained unaffected by small variations of these parameters. The results from the two columns indicated that there is no significant difference between the results from the two columns.

3.6. Limit of Detection and Limit of Quantitation. The LOD and LOQ were found to be 1.08 and 3.28 μg mL^{-1}, respectively, for PCM, 1.43 and 4.34 μg mL^{-1}, respectively, for CHZ and 0.30 and 0.92 μg mL^{-1}, respectively, for NIM.

3.7. Specificity. There is no peak interference of blank and placebo at the retention time of PCM, CHZ, and NIM which indicates that the method is specific for the analysis in their pharmaceutical dosage form. The specificity of the method is illustrated in Figure 4 where complete separation of PCM, CHZ and NIM was noticed. The average retention time (t_R) ± SD for PCM, CHZ, and NIM was found to be 2.69 ± 0.02, 4.61 ± 0.01, and 9.55 ± 0.02 min, respectively, for six replicates. Tailing factor for peaks of PCM, CHZ, and NIM was less than 2 ($T \leq 2$) and resolution was satisfactory (Rs ≥ 2). The peaks obtained were sharp and have clear baseline separation.

3.8. System Suitability. System suitability parameters including theoretical plates, peak asymmetry (T), capacity factor (K'), selectivity (α), and resolution (Rs) between PCM, CHZ, and NIM peaks were calculated and summarized in Table 4.

3.9. Accuracy. As shown from the data in Table 5, satisfactory recovery % with small relative standard deviations (% RSD) were obtained at various added concentrations. The results indicate the method is highly accurate for simultaneous determination of the three drugs.

Table 3: Robustness evaluation of the method ($n = 6$).

Factor	Level	Retention time (t_R)			Asymmetry (T)		
		PCM	CHZ	NIM	PCM	CHZ	NIM
(A) Flow rate (mL min^{-1})							
1.1	−1	2.700	4.625	9.582	1.24	1.07	1.12
1.2	0	2.702	4.612	9.545	1.18	1.05	1.09
1.3	+1	2.697	4.595	9.461	1.15	1.02	1.08
Mean ± S.D		2.699 ± 0.04	4.610 ± 0.02	9.529 ± 0.06	1.19 ± 0.04	1.05 ± 0.02	1.10 ± 0.06
(B) Percentage of acetonitrile in the mobile phase (v/v)							
44	−1	2.710	4.650	9.567	1.14	1.00	1.11
45	0	2.702	4.612	9.545	1.18	1.05	1.09
46	+1	2.690	4.596	9.525	1.10	1.02	1.08
Mean ± S.D		2.700 ± 0.01	4.619 ± 0.03	9.546 ± 0.02	1.13 ± 0.04	1.03 ± 0.03	1.09 ± 0.02
(C) Columns from different manufacturers							
Hypersil GOLD C_{18}		2.702	4.612	9.545	1.18	1.05	1.09
HiQ-Sil HS C_{18}		2.697	4.620	9.550	1.12	1.18	1.08
Mean ± S.D		2.699 ± 0.03	4.616 ± 0.01	9.547 ± 0.01	1.19 ± 0.03	1.05 ± 0.08	1.10 ± 0.02

Table 4: System suitability parameters for PCM, CHZ, and NIM by the proposed HPLC method.

Parameters	PCM	CHZ	NIM	Reference values
Theoretical plates (N)	6803.98	11021.67	12921.34	$N > 2000$
Peak asymmetry (T)	1.18	1.05	1.08	$T \leq 2$
Capacity factor (K')	0.22	1.08	3.34	$1 < K' < 10$
Selectivity (α)[a]	—	1.71	2.07	$\alpha > 1$
Resolution (Rs)[a]	—	12.50	19.03	Rs ≥ 2
HETP (H)[b]	0.04	0.02	0.02	—

[a]With respect to previous peak.
[b]HETP (height equivalent to theoretical plate).

Table 5: Accuracy studies for the determination of PCM, CHZ and NIM ($n = 6$).

	Excess drug added to the analyte (%)	Theoretical Content (μg mL^{-1})	Measured conc. ± SD	Recovery (%)	% R.S.D.	S.E.
PCM	80	40.95	41.27 ± 0.37	100.78	0.89	0.36
	100	45.50	45.10 ± 0.45	99.12	0.99	0.40
	120	50.05	49.95 ± 0.25	99.80	0.50	0.21
CHZ	80	47.25	47.45 ± 0.85	100.04	1.79	0.73
	100	52.50	52.15 ± 0.97	99.33	1.86	0.76
	120	57.75	58.05 ± 0.75	100.50	1.29	0.53
NIM	80	12.60	12.55 ± 0.15	99.60	1.19	0.49
	100	14.00	14.05 ± 0.25	100.35	1.77	0.72
	120	15.40	15.25 ± 0.10	99.02	0.65	0.27

3.10. Analysis of a Marketed Pharmaceutical Dosage Form. Using the proposed chromatographic method, assay of PCM, CHZ, and NIM in their tablets NICIP MR (label claim: 325 mg PCM, 375 mg CHZ, and 100 mg NIM per tablet, B. No. AH0313, Cipla Pharmaceuticals Ltd.) was carried out. Satisfactory results were obtained for all three drugs in a good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets. The recovery %± RSD % of six replicate determinations was 99.64 ± 0.57 for PCM, 99.97 ± 0.75 for CHZ, and 99.93 ± 0.39 for NIM.

4. Conclusion

The developed HPLC technique is precise, specific, robust, and accurate. Statistical analysis proves that the method is suitable for routine analysis of PCM, CHZ, and NIM in pharmaceutical dosage form.

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