Estimation of four drugs: Ambroxol hydrochloride, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Paracetamol by RP-HPLC in tablet dosage form

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Abstract
A simple, specific, precise, accurate and economic isocratic RP-HPLC method was developed for the simultaneous determination of Ambroxol hydrochloride, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Paracetamol in bulk and tablet dosage form. A reverse phase Nucleosil C_{18} column (250 mm x 4.6 mm x 5 µm) with mobile phase consisting of Methanol: Sodium Phosphate dibasic anhydrous Buffer (65:35 V/V) having (pH of buffer 7.0 ± 0.02 adjusted with ortho phosphoric acid) was used. The flow rate was 1.0 mL/min and the effluents were monitored at 230 nm. The retention times of Paracetamol, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Ambroxol hydrochloride were found to be 3.117, 4.925, 6.217 and 12.308 min. respectively. The method was validated in terms of linearity, range, specificity, accuracy, precision and robustness. The proposed method was successfully applied for the estimation of paracetamol, levocetirizine hydrochloride, phenylephrine hydrochloride and ambroxol hydrochloride in combined tablet dosage form.

Keywords: Paracetamol (PCM), Levocetirizine hydrochloride (LEVO), Phenylephrine hydrochloride (PHEN), Ambroxol hydrochloride (AMB), Isocratic; RP-HPLC.

Introduction
Paracetamol is analgesic and antipyretic chemically it is N-(4-hydroxyphenyl) acetamide. Phenylephrine hydrochloride chemically is (1R)-1-(3hydroxy-phenyl)-2-(methylamino) ethanol hydrochloride and is sympathomimetic (descongestants). Levocetirizine hydrochloride chemically 2-[2-[4-[(R)-(4 chlorophenyl)-phenylmethyl] piperazin-1yl] ethoxy] acetic acid; dihydrochloride and is antihistaminic and Ambroxol hydrochloride is chemically trans- 4-[(2-amino-3, 5 dibromobenzyl) amine] cyclohexanol hydrochloride and is used as expectorants or mucolytic.[1] Structural formulas of PCM, LEVO, PHEN and AMB are given in Fig. 1.[2, 3] The combined dosage form of PCM, LEVO, PHEN and AMB are more effective in controlling common cold and severe allergic cases than individual drugs. The Literature survey revealed that this combination is not official in any Pharmacopoeia but there are several methods that have been reported to determine PCM, LEVO, PHEN and AMB as individual or in combination with other drugs, such as UV, UPLC, LC-MS, GC-MS, LC-MS/MS and HPLC with UV/PDA detection.[4-22] For estimation of these four drugs combination, two methods are available such as RP-HPLC and UV.[23,24] Literature survey reveals that no isocratic elution method reported yet for the determination of Ambroxol hydrochloride, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Paracetamol in combine Dosage form.

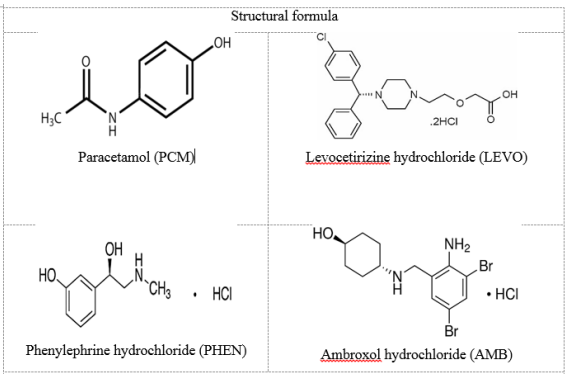

Fig. 1: The structures of paracetamol (PARA), lecocetirizine hydrochloride (LEVO), phenylephrine hydrochloride (PHEN) and Ambroxol hydrochloride (AMB)

Experimental Condition
Chemicals and reagents: Working standards of pharmaceutical grade Paracetamol and Ambroxol hydrochloride were present in our college. Levocetirizine hydrochloride and Phenylephrine hydrochloride were gifted by Vaikunth Chemicals (PVT.) LTD. (Ankleshwar, India) and Darshan Pharmachem (PVT.) LTD. (Ankleshwar, India). Fixed dose combination tablet Cheston cold total (Cipla) containing 5 mg Levocetirizine hydrochloride, 5 mg Phenylephrine hydrochloride, 30 mg Ambroxol hydrochloride and 325 mg Paracetamol was purchased from local market. All the chemicals used were of HPLC grade.

Equipment and Chromatographic conditions: The HPLC system consisted of Shimadzu LC-2010CHT

pump serial dual plunger, UV-detector, autosampler; data were acquired and processed by making use of CLASS-VP software (all equipments from Shimadzu). The chromatographic separations were carried out on a reverse phase Nucleosil C18 analytical column (250mm x 4.6mm, 5 µm).

Preparation of standard stock and sample solution

Preparation of standard stock solution: Weigh accurately about 5 mg of LEVO working standard in 25 ml volumetric flask, add about 10 ml of diluent (methanol : water 50:50% v/v) to dissolve with the aid of ultrasound for about 5 minutes with occasional shaking and dilute it with diluent upto the mark to get the concentration 200 µg/ml. Similarly prepare standard solution of PHEN (200 µg/ml). For preparation of AMB stock solution, Weigh 12 mg AMB in 20 ml volumetric flask, add about 10 ml diluent to dissolve it with the aid of ultrasound for about 5 minutes and make volume with diluent up to the mark (600 µg/ml). And weigh accurately 13 mg of PCM standard in 20 ml volumetric flask; add about 10 ml of diluent to dissolve it with the aid of ultrasound for 5 minutes. Then withdraw 1 ml of PHEN and LEVO and 2 ml of AMB from their stock solution and then dilute it up to mark with Diluent. Final concentration of LEVO, PHEN, AMB and PCM standard was 10:10:60:650 µg/ml respectively.

Preparation of Sample for analysis of formulation: For preparation of sample solution of pharmaceutical mixture twenty tablets (Cheston cold total Tablet) were weighed and powdered finely. Tablet powder equivalent to 325 mg of PCM, 30 mg of AMB, 5 mg of PHEN and 5 mg of LEVO was transferred in 50 ml of volumetric flask. Add about 30 ml of diluent to dissolve with the aid of ultrasound for about 20 minutes with occasional shaking and make volume with diluent. Filter the solution through 0.45 µm membrane filter. Discard first 5 ml of the filtrate than dilute 1ml of this filtrate solution in 10 ml of volumetric flask and make upto mark with diluent. Final concentration of LEVO, PHEN, AMB and PCM was 10:10:60:650 µg/ml respectively. The resultant mixture was subjected to HPLC analysis in developed chromatographic conditions.

Chromatographic condition: The Column was **Nucleosil** (250 mm × 4.6 mm × 5 µm) packed with end-capped octa- decylsilyl (C_{18}) silica gel. Mobile phase was mixture of Methanol and 10 mM Sodium Phosphate dibasic anhydrous Buffer (pH 7, adjusted with ortho phosphoric acid) in ratio of (65:35 % v/v) at isocratic mode. Flow rate of mobile phase was kept at the flow of 1 ml/min. Eluents were detected at 230 nm. The mobile phase was filtered with 0.45 µm membrane filter and degassed before use. The injection volume was 10 µl and all analytes were analysed at Column temperature 40 °C.

Analytical Method Validation[25]

Linearity: In Linearity 10 mg of LEVO, similarly prepared solution of PHEN, 60 mg of AMB and 650 mg of PCM in 100 ml of volumetric flask, then withdraw 0.25, 0.5, 0.75, 1, 1.25 and 1.5 ml separately into 10 ml of volumetric flask. Final concentration of LEVO, PHEN, AMB and PCM were 2.5:2.5:15:162.5 µg/ml, 5:5:30:325 µg/ml, 7.5:7.5:45:487.5 µg/ml, 10:10:60:650 µg/ml, 12.5:12.5:75:812.5 µg/ml and 15:15:90:975µg/ml respectively.

Specificity: In specificity diluent and solution of LEVO, PHEN, AMB and PCM were injected in HPLC system following the test conditions; the chromatograms were recorded and measured the responces of peaks were noted for interference of the excipients between sample solutions and blank. Final concentration range of LEVO, PHEN, AMB and PCM was 10:10:60:100µg/ml respectively.

Limit of Detection and Limit of Quantification: As per ICH guideline, limit of detection and quantification of developed method were calculated from the standard deviation of y-intercept and average of slope of the calibration curve of Levocetirizine hydrochloride, Phenylephrine hydrochlorid, Ambroxol hydrochloride and Paracetamol using the formula: Limit of Detection = 3.3* σ/S, Limit of Quantification = 10* σ/S Where, "σ" is the Standard deviation of intercept of 5 calibration curve, "S" is average of slope of 5 calibration curve.

Accuracy: The percentage recovery was performed by adding a known quantity of pure standard drug into the pre-analyzed sample. Accuracy of method was ascertained by performing recovery at 3 levels in triplicates at 80% (585:9:9:54 µg/ml), 100% (650:10:10:60 µg/ml) and 120% (715:11:11:66µg/ml) for PCM, LEVO, PHEN and AMB respectively. The results were expressed as percentage.

Precision

Repeatability (n= 6) was carried out for 100.0% of the test concentration. In the present case, concentrations at 650, 10, 10 and 60 µg/ml for PCM, LEVO, PHEN and AMB respectively were used. Inetrday and Intraday precision (n=3) was performed on different days and same day by performing at 3 levels in triplicates at 80% (585:9:9:54 µg/ml), 100% (650:10:10:60 µg/ml) and 120% (715:11:11:66 µg/ml) for PCM, LEVO, PHEN and AMB respectively. Results are reported in terms of % RSD of peak area.

Robustness

Robustness of the developed method was evaluated at concentration 10 µg/ml LEVO, 10 µg/ml PHEN, 60 µg/ml AMB and 650 µg/ml PCM by deliberate change in different parameters like flow rate (1 ml/min. ± 0.1 ml/min.), pH (7 ± 0.1), column temperature (40 °C ± 1) showed % RSD of peak area was calculated

Result and Discussion

Method optimization: An isocratic RP-HPLC method was optimized for determination of all four drugs. Satisfactory results were achieved by using 65:35 % v/v Methanol: 10mm Sodium Phosphate dibasic anhydrous Buffer (pH 7, adjusted with Ortho phosphoric acid) at flow rate of 1 ml/min followed by detection at 230 nm. System suitability parameters are acceptable. Fig. 2 shows the HPLC Chromatogram for simultaneous determination of standard mixture of PCM, LEVO, PHEN and AMB obtained through the optimized variables in accordance with the features described above. Table 1 shows system suitability parameters such as retention time, area, resolution and asymmetry obtained for optimal chromatographic conditions.

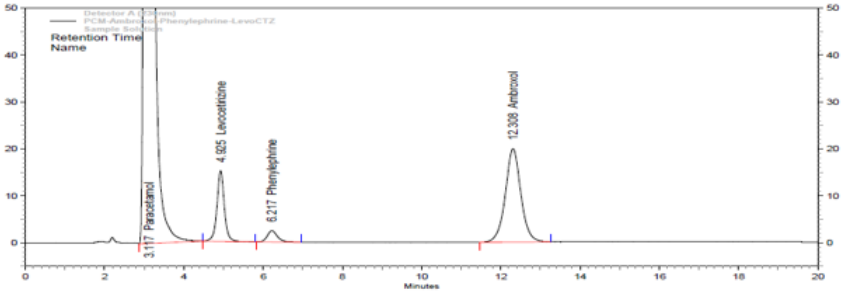

Figure 2: HPLC Chromatogram of Optimize condition of PCM, LEVO, PHEN and AMB

Table 1: System suitability Parameters

Name of Drug	Retentiont ime (min)	Area (µV*Second)	Theoritical plates	Resolution	Asymmetry
Paracetamol	3.117	18146360	3744.21	0.00	0.98
Levocetirizine HCl	4.925	195016	3709.54	6.86	1.06
Phenylephrine HCl	6.217	43491	2978.98	3.32	1.27
Ambroxol HCl	12.308	543043	4913.25	10.52	1.02

Method validation

Linearity: For linearity, six concentrations were chosen ranging from 25% to 150% of the target analyte concentrations. Linear responses were obtained in concentration range of 162.5 - 975µg/ml for PCM, 2.5–15µg/ml for LEVO and PHEN and 15–90µg/ml for AMB. Fig. 3 shows the linearity overlay chromatogram of calibration curve and Fig. 4 shows calibration graph obtained by plotting peak area versus concentration of standard drugs PCM, LEVO, PHEN and AMB.

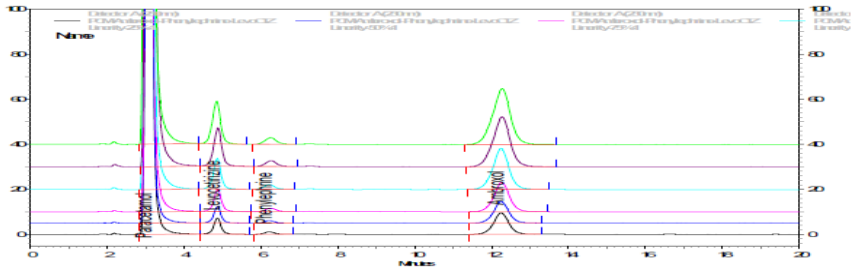

Fig. 3: Overlay graph of Calibration curve

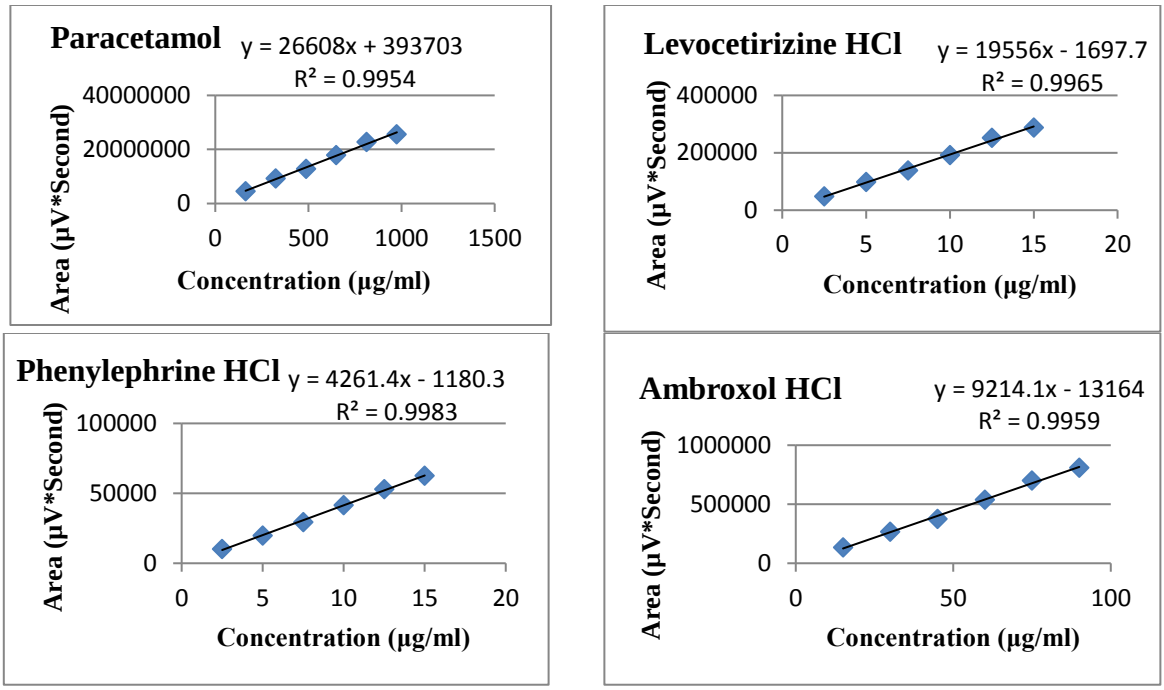

Fig 4: Calibration curve of Paracetamol, Levocetirizine HCl, Phenylephrine HCl and Ambroxol HCl

Specificity: % Interference of excipients were found to be less than 0.5 for PCM, LEVO, PHEN and AMB, so method was found to be specific. Specificity studies for PCM, LEVO, PHEN and AMB are shown below in Fig. 5 and results are tabulated in Table 2.

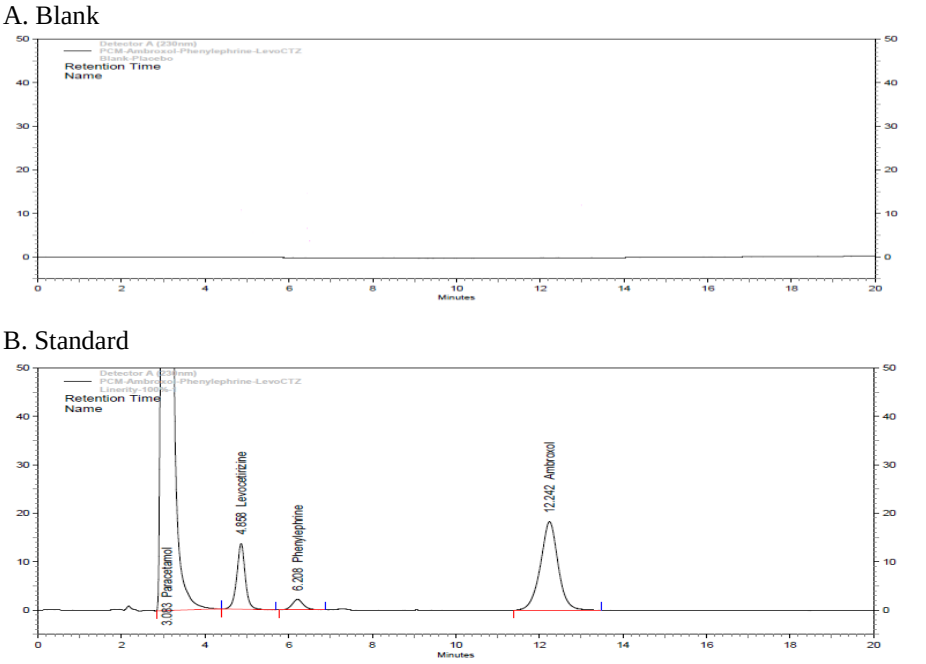

Fig. 5: Chromatogram of blank and Standard for specificity

Table 2: Resultant Data of % interference in Specificity

Sr. No.	Before Spiking Without Excipients (µg/ml)				After Spiking With Excipients (µg/ml)				% Interference			
	PCM	LEVO	PHEN	AMB	PCM	LEVO	PHEN	AMB	PCM	LEVO	PHEN	AMB
1	650.51	9.90	10.02	59.72	651.58	9.92	10.04	59.9	0.164	0.17	0.164	0.297
2	651.21	9.90	10.02	59.73	652	9.93	10.05	60.04	0.122	0.326	0.255	0.512
3	650.45	9.90	10.05	60.06	651	9.89	10.08	60.25	0.085	-0.141	0.286	0.312
4	651.55	9.90	9.95	59.99	651.8	9.91	9.99	60.02	0.038	0.132	0.361	0.055
5	650.87	9.91	10.00	60.10	652	9.91	10.02	60.01	0.174	0.045	0.225	-0.155
Mean									**0.117**	**0.107**	**0.258**	**0.204**

Limit of Detection and Limit of Quantification: Limit of detection and quantification of developed method were calculated from the standard deviation of y-intercept and average of slope of the calibration curve of LEVO, PHEN, AMB and PCM. Table 3 shows the resultant data of LOD and LOQ.

Table 3: Resultant Data of LOD and LOQ

Name	Limit of Detection S/N ratio	Limit of Quantification S/N ratio
Paracetamol	3.348	10.148
Levocetirizine HCl	0.119	0.361
Phenylephrine HCl	0.523	1.587
Ambroxol HCl	0.923	2.797

Accuracy: Accuracy of the method was determined using standard addition method and expressed as % recovery. Accuracy was assessed by spiking of LEVO, PHEN, AMB and PCM at different level (80%, 100% and 120%) of target concentrations of 5µg/ml LEVO, 5µg/ml PHEN, 30 µg/ml AMB and 325µg/ml PCM were injected in developed chromatographic conditions in triplicate (n=3). Table 4 shows the result of Accuracy.

Table 4: Result of Accuracy for Paracetamol, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Ambroxol hydrochloride

Sr. No.	Level	Targated Conc. (µg/ml) P:L:Phe:A	Amount Added (µg/ml)	Total Conc. (µg/ml)	Conc. of PCM (µg/ml)	Conc. of Levo. HCl (µg/ml)	% recovery of PCM	% recovery of Levo
1	80%	325:5:5:30	260:4:4:24	585:9:9:54	583.74	8.8996	99.78	98.88
		325:5:5:30	260:4:4:24	585:9:9:54	584.97	9.0654	100.00	100.73
		325:5:5:30	260:4:4:24	585:9:9:54	582.19	8.9111	99.52	99.01
2	100%	325:5:5:30	325:5:5:30	650:10:10:60	662.63	9.8968	101.94	98.97
		325:5:5:30	325:5:5:30	650:10:10:60	643.83	9.9314	99.05	99.31
		325:5:5:30	325:5:5:30	650:10:10:60	650.49	9.9490	100.07	99.49
3	120%	325:5:5:30	390:6:6:36	715:11:11:66	715.62	10.9473	100.09	99.52
		325:5:5:30	390:6:6:36	715:11:11:66	713.80	10.9519	99.83	99.56
		325:5:5:30	390:6:6:36	715:11:11:66	714.47	11.1778	99.93	101.62

Precision: Repeatability (n=6) was carried out for 100.0% of the test concentration. In the present case, concentrations at 650, 10, 10 and 60µg/ml for PCM, LEVO, PHEN and AMB respectively were used. Inetrday and Intraday precision (n=3) was performed on different days and same day using concentration of 5µg/ml for LEVO, 5µg/ml for PHEN, 30µg/ml for AMB and 325µg/ml for PCM respectively. The inter-day and intra-day precision (% RSD) was found to be less than 2% RSD reveals that the proposed method provides an acceptable result of intraday and interday precision as shown in Table 5 and 6.

Table 5: Result of Repeatability for Paracetamol, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Ambroxol hydrochloride

Sr. no.	PCM	LCT	PHEN	AMB
Conc.	**650 µg/ml**	**10 µg/ml**	**10 µg/ml**	**60 µg/ml**
1	18073397	194832	43490	549254
2	18675541	194840	41260	546494
3	18016755	194235	42554	543586
4	18065246	194621	43047	552167
5	18130213	197596	43034	562861
6	18357530	194773	43245	554111
Mean	18219780	195149.5	42771.67	551412.2
SD	253473.2	1219.781	802.1458	6767.927
RSD	**1.391198**	**0.62505**	**1.875414**	**1.227381**

Table 6: Result of Interday and Intraday Precision for Paracetamol, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Ambroxol hydrochloride

Sr No.	Conc. (%)	Conc. (µg/ml)	Name of Drugs	Interday Precision (n=3)		Intraday Precision (n=3)	
				Mean Area ± SD	%RSD	Mean Area ± SD	%RSD
1	80	585	PCM	15716361.22 ± 56427	0.36	15538075.6 ± 252464.1	1.62
2		9	LCT	172183.67 ± 1873.439	1.08	164784.44 ± 2616.196	1.59
3		9	PHEN	33745 ± 458.3657	1.35	34395.1111 ± 339.6481	0.99
4		54	AMB	488351.33 ± 5686.265	1.16	491458.444 ± 3377.115	0.69
5	100	650	PCM	18457136.67 ± 2080.921	0.41	18586709.44 ± 229115.9	1.23
6		10	LCT	195398.89 ± 2080.921	1.06	194865.22 ± 1832.523	0.94
7		10	PHEN	41653.44 ± 291.9862	0.7	41332.7778 ± 259.6267	0.63
8		60	AMB	540526.33 ± 6221.879	1.15	548168.667 ± 7359.516	1.34
9	120	715	PCM	20785603.11 ± 157200.5	0.75	20406131.78 ± 243246.2	1.19
10		11	LCT	226984.44 ± 1436.917	0.63	223926.33 ± 2159.837	0.96
11		11	PHEN	49626.44 ± 293.9712	0.59	49475.4444 ± 284.0377	0.57
12		66	AMB	670271.44 ± 6229.258	0.92	651712.889 ± 3996.695	0.61

Robustness: Robustness of the developed method evaluated by deliberate change in different parameters like flow rate, pH, column temperature showed % RSD of peak area less than 2, indicating that the method was robust. Table 7 shows resultant data of robustness.

Table 7: Robustness study for Paracetamol, Levocetirizine hydrochloride, Phenylephrine hydrochloride and Ambroxol hydrochloride

Parameter	Change	PCM		LCT	
		Mean Area ± S.D (n=3)	% RSD	Mean Area ± S.D (n=3)	% RSD
Flow rate (ml/min.)	0.9	18016880 ± 56175.2	0.31179	192187 ± 483.844	0.25176

	1	17762931± 249872	1.4067	181608 ± 517.786	0.28511
	1.1	18086192± 62255.8	0.34422	197336 ± 658.31	0.3336
pH	6.9	18049323± 56360.6	0.31226	187249 ± 513.943	0.27447
	7	18198761± 43871.2	0.24107	184433 ± 865.945	0.46952
	7.1	18120526± 37628.2	0.20766	187856 ± 616.599	0.32823
Column Temp. (°C)	39	18071306± 88180.4	0.48796	189186 ± 577.981	0.30551
	40	18189969± 18711.7	0.10287	183502 ± 1123.93	0.61249
	41	18096131± 129124	0.71354	193227 ± 886.608	0.45884

Analysis of formulation: The tablet formulation Cheston cold Total tablet analyzed using the developed method, showed separated peaks. The % Potency was achieved 100.97 for PCM, 101.58 for LEVO, 100.94 for PHEN and 100.25 for AMB. The quantitative results of this assay are summarized in Table 8.

Table 8: Result of % Potency

Name of Drug	Label Claim (mg)	Area of Standard	Area of Sample	Potency
Paracetamol	325	17970507	18146360	100.97%
Levocetirizine HCl	5	191968	195016	101.58%
Phenylephrine HCl	5	41534	43491	100.94%
Ambroxol HCl	30	537127	543043	100.25%

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conclusion

The Isocratic RP-HPLC method developed and validated for PCM, LEVO, PHEN and AMB was found to be simple, specific, precise, accurate, rapid, robust and economical. Separation of four drugs done by good resolution within a short analysis time of less than 15 min. The method was found to be specific and accurate as % interference were less than 0.5 and accuracy was in the range of 98 – 102 % for all four drugs. % RSD for all parameters were found to be within the limit. This indicates the result and assay obtained by this method are in good agreement. Thus the method developed can be used for the routine analysis of PCM, LEVO, PHEN and AMB in laboratories as well as industries for quality control purpose.

Acknowledgement

The authors are thankful to Shree Dhanvantary Pharmaceutical Analysis and Research Center, Kim, Surat, Gujarat, India for providing necessary facilities to carry out the research work.

Declaration(s) of Interest

Authors have no declaration(s) and conflict(s) of interest.

Author's Contribution

Principal author: Planned the experimental setup, performed lab work, interpreted data and wrote the manuscript.

Co- author's Contribution: Supervised the development of work and helped in the evaluation of the manuscript. Both authors read and approved the final manuscript.

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