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DEVELOPMENT AND VALIDATION OF ROBUST RP-HPLC METHOD FOR QUANTITATION OF DEXTROMETHORPHAN HBr, PHENYLEPHRINE HCl, CHLORPHENIRAMINE MALEATE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT-

Dextromethorphan hydrobromide suppresses cough reflex centrally, Phenylephrine HCl constricts nasal blood vessels to ease congestion, and Chlorpheniramine maleate relieves allergy symptoms. In that, mobile phase comprised a phosphate buffer at pH 3.2, Acetonitrile and Methanol. Detection was achieved using a UV-Visible detector at 220 nm, with a flow rate maintained at 1.0 ml/min. Rigorous validation of the method included assessments for linearity, precision, accuracy, specificity, robustness, and statistical analysis of the obtained data. The calibration curve exhibited linearity within a concentration range of 1–210 µg/ml, and a regression coefficient value of 0.9999, 0.9997, 0.9992. The limit of detection and limit of quantification were determined as 0.011, 0.162, 0.102 and 0.034, 0.491, 0.310 µg/ml, respectively. Precision assessment revealed a % RSD (Relative Standard Deviation) of less than 1%, demonstrating the methods precision. Also, it indicated a recovery range of 80 to 120 %, with a % RSD less than 1%. This thorough evaluation not only underscores the accuracy and precision of the method but also highlights its sensitivity, solidifying its status as a reliable approach for the precise quantitative analysis of Dextromethorphan HBr, Phenylephrine HCl, Chlorpheniramine Maleate in commercial formulations, distinct peak with clear resolution and sensitive RP-HPLC gradient method was developed and validated.

KEYWORDS-

Estimation, Dextromethorphan HBr, Phenylephrine HCl, Chlorpheniramine Maleate, RP-HPLC.

INTRODUCTION-

Dextromethorphan hydrobromide [3-Methoxy-17-methylmorphinan hydrobromide mono hydrate], Upon oral consumption, it swiftly enters the bloodstream from the stomach, transforming into the less active dextrorphan form. Its effects persist for roughly three to eight hours following ingestion. Dextromethorphan HBr, a cough suppressant, exhibits low solubility in water but is soluble in methanol and ethanol [1].

Dextromethorphan functions by obstructing N-methyl-D-aspartate (NMDA) receptors, pivotal in pain transmission and the cough reflex, thereby diminishing the coughing sensation. Furthermore, it impedes serotonin reuptake and interacts with sigma-1 receptors. Its efficacy in cough suppression predominantly stems from its antagonism of NMDA receptors. Conversely, histamine binding to H1 receptors prompts allergy symptoms such as itching, sneezing, and a runny nose.[2]

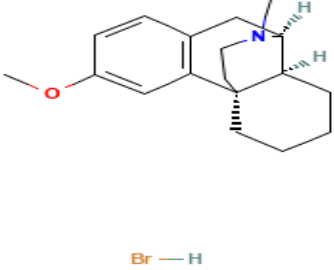
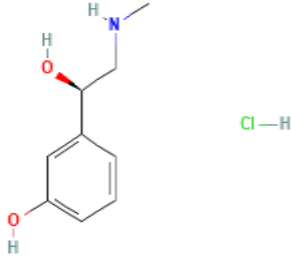
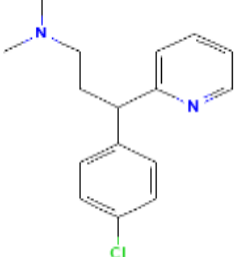
Phenylephrine HCl (R)-3-[-1-hydroxy-2-(methylamino) ethyl], it is very soluble in water and serves as a decongestant and undergoes notable alterations in the stomach and liver due to monoamine oxidase (MAO) enzymes, leading to decreased efficacy. Phenylephrine acts as a decongestant, when orally administered, its efficacy is compromised due to enzymatic activity in the stomach and liver, resulting in only about 38% of its intended effect, also diminishes vessel engorgement and mitigates fluid leakage into nasal tissues. Consequently, nasal congestion is alleviated, facilitating easier breathing through the nose. Which induces vasoconstriction by activating alpha-1 receptors, thereby diminishing blood flow to the nasal mucosa, and alleviating nasal congestion, ultimately facilitating easier breathing.[3]

Chlorpheniramine maleate 3-(4-Chlorophenyl)-N, N-dimethyl-3-(pyridin-2-yl)-propan-1-amine diethyl (Z)-but-2-enedioate, serves as an antihistamine commonly found in cold and allergy medications. Its mechanism of action involves the inhibition of histamine's effects, providing relief from symptoms such as a runny nose and itchy eyes. Soluble in water and is effective against histamine-related issues. Chlorpheniramine maleate, categorized as an antihistamine, is present in various cold and allergy remedies. [4].

This method, following ICH guidelines, ensures reliability and precision of these drugs. Our study introduces significant parameters including the mobile phase, column selection, and temperature control. These modifications were strategically implemented to enhance the assay

performance, surpassing existing methodologies. Hence, we opted for Reverse Phase High-Performance Liquid Chromatography to determine Dextromethorphan HBr, Phenylephrine HCl, and Chlorpheniramine maleate in marketed syrup.

Fig 01- The Chemical Structure of all Active material involved in this method development

		
<p>Chemical Structure of Dextromethorphan HBr</p>	<p>Chemical Structure of Phenylephrine HCl</p>	<p>Chemical Structure of Chlorpheniramine maleate</p>

MATERIALS AND METHODS-

Reagents and Chemicals –

The reference standard for Dextromethorphan hydrobromide was provided by Onriro Chemicals Pvt. Ltd., for Phenylephrine HCL was provided by Neha Pharma Pvt. Ltd and for Chlorpheniramine maleate were provided by Anusha Associates. The pharmaceutical dosage cough syrup, contain Dextromethorphan HBr, Phenylephrine HCL and Chlorpheniramine maleate was obtained from a local pharmacy. All chemicals utilized in the study were of HPLC grade, with Methanol, Acetonitrile, water, and Orthophosphoric Acid these were sourced from Merck Life Science, Pvt. Ltd, while 1-octane sulphonic acid sodium salt anhydrous 99% by Loba Chemie, Pvt, Ltd and Triethyl amine were acquired from Sigma-Aldrich Pvt. Ltd.

Instrumentation and Chromatographic Conditions-

The experimental configuration utilized on Vanquish Core thermo scientific HPLC System, highlighting a Quaternary pump included a detection with Diode Array Detector (DAD). The chromatographic separation was conducted employing a ZORBAX extend- octa decyl silane C-18 column (250 mm* 4.6 mm* 5µm) 80A°. The system was smoothly incorporated with

Chromeloan-7.3.9.0 Software. Mobile phase containing buffer 3.2: acetonitrile (100% ACN): methanol, the diluent was made with buffer: (100% ACN) Acetonitrile with 50:50 ratio. The mobile phase underwent filtration and sonication before being delivered at a flow rate of 1.0 mL/min. The detection occurred at a wavelength of 220 nm. During analysis, a 20 μ L injection volume was utilized.

Preparation of Standard Solution-

A combined mixed standard solution was prepared in the diluent from the standard solution to obtain solutions containing 150, 50, 20 μ g/ml of Dextromethorphan hydrobromide, Phenylephrine HCl, and Chlorpheniramine maleate, respectively. They were injected six times. The peak area of each was plotted versus concentration, and calibration curves were structured.

Preparation of Test Solution-

Sample taken make appropriate dilutions and syrup was dissolve in 100 ml of volumetric flask with diluent. The mixture was sonicated for 15 min. Subsequently, the solution was thoroughly shaken and filtered through Whatman filter paper. a subsequent dilution step was carried out by using buffer 3.2 and acetonitrile in a 50:50, v/v ratio resulting in a 15, 5, 2 μ g/ml of Dextromethorphan HBr, Phenylephrine HCl, and Chlorpheniramine maleate solution. Finally sample solutions were filtered with 0.45 μ m syringe filtration disk to the vials for injection into HPLC system.

ANALYTICAL METHOD DEVELOPMENT-

During experimental trials, a systematic exploration of various chromatographic conditions was conducted, encompassing the utilization of different columns, namely the Sunniest C18 column (250 mm \times 4.6 mm, 5 μ m) and the Zorbax Extend C18 column (250 mm \times 4.6 mm, 5 μ m).

Gradient elution techniques were employed, employing solvent compositions comprising a buffer with a pH of 3.2 adjusted by ortho-phosphoric acid (OPA) in conjunction with acetonitrile (ACN). Subsequent modifications were implemented, introducing methanol (MeOH) into the solvent gradient in addition to the buffer of pH 3.2 adjusted by OPA and ACN. illustrated in Fig 02 a. Further investigation was undertaken to optimize chromatographic conditions, including adjustments to the column temperature, with subsequent trials conducted

at 20°C and 25°C. Despite efforts to improve peak shape by elevating the column temperature to 40°C, satisfactory extraction of the active pharmaceutical ingredient (API) was not achieved.

To address the challenge of optimal API extraction from the formulation, experimentation encompassed the utilization of various diluents. Notably, combinations of ACN and buffer at pH 3.2 in ratios of 50:50 and 60:40 were employed during the dilution process. However, the chromatographic results revealed merging peaks for Chlorpheniramine maleate and Dextromethorphan HBr, accompanied by inadequate resolution.

Ultimately, The Zorbax Extend C18 column (250 mm×4.6 mm, 5 µm, 80Å) was identified as the optimal chromatographic medium for the identification and quantification of Dextromethorphan HBr, Phenylephrine HCl, and Chlorpheniramine Maleate. Employing gradient elution with a combination of buffer pH 3.2 (Phase I), acetonitrile (ACN) (Phase II), and methanol (MeOH) (Phase III), alongside a diluent comprising ACN and buffer at pH 3.2 in a 50:50 ratio, yielded optimal results in terms of resolution, peak shape, and sensitivity. The selected gradient program exhibited the following composition over time: 0 min (70:20:10%), 5 min (70:20:10%), 10 min (65:10:25%), 15 min (55:30:15%), 20 min (50:30:20%), 25 min (30:50:20%), 30 min (20:60:20%), 45 min (70:20:10%), 50 min (70:20:10%).

Spectrophotometric detection at 220 nm was chosen for monitoring all three active pharmaceutical ingredients (APIs), owing to its demonstrated highest sensitivity towards compound, illustrated in Fig 02 b. Throughout the experimental trials, consistency was maintained in the sample volume for analysis (20 µl), temperature (30°C), and flow rate (1.0 mL/min), respectively.

Fig 02. a - trail – 1 This trial was performed in mobile phase combination of buffer (pH 3.2): acetonitrile (ACN)

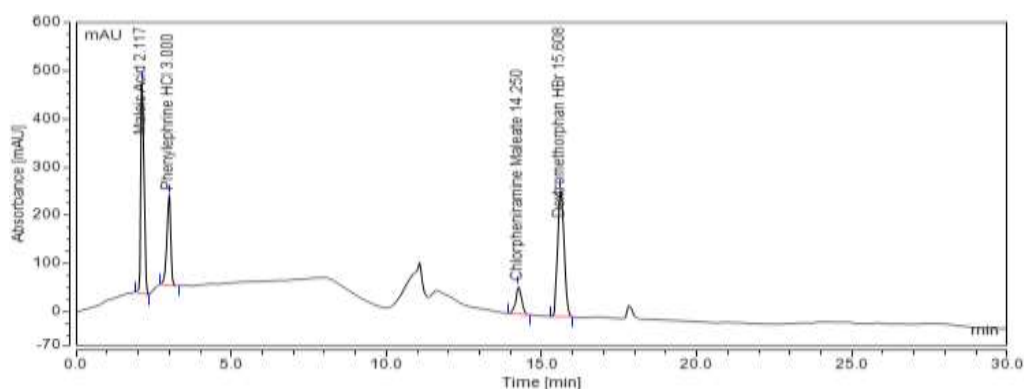
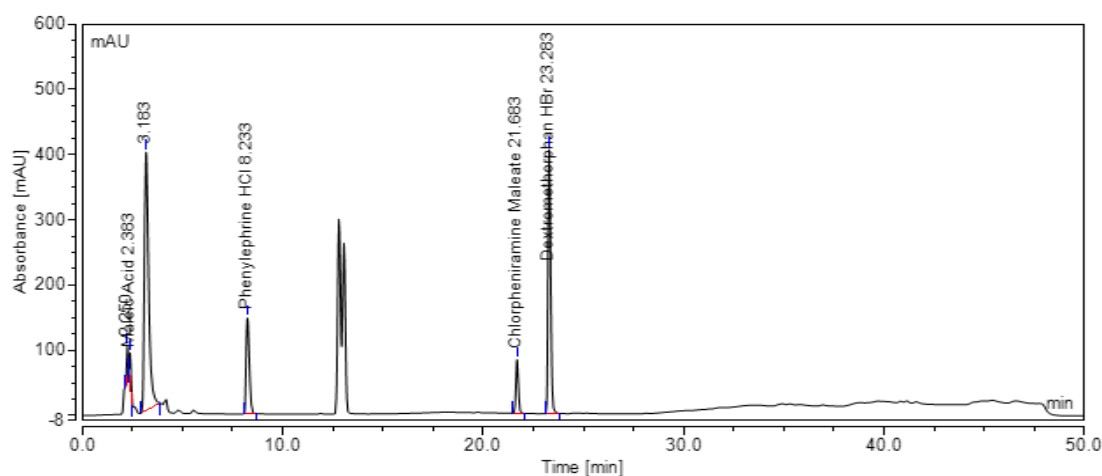


Fig 02. b - trail – 2 This trial was performed in mobile phase combination of buffer (pH 3.2): acetonitrile (ACN): methanol (MeOH)



ANALYTICAL METHOD OPTIMIZATION -

Several experiments were conducted to explore various columns, mobile phases, and pH levels with the aim of determining the optimal chromatographic conditions that exhibit desirable peak characteristics. Among the array of columns tested, the Sunniest C18 column (250 mm×4.6 mm, 5 μm) and the Zorbax Extend C18 column (250 mm×4.6 mm, 5 μm, 80Å), combined with a mobile phase composed of gradient elution using combination of Buffer pH 3.2 (Phase I), acetonitrile (ACN) (Phase II), and methanol (MeOH) (Phase III) and diluent comprising ACN and buffer at pH 3.2 in a 50:50 ratio. Implementing gradient program with maintaining a constant flow rate of 1 mL/min was determined to provide satisfactory peak symmetry for Dextromethorphan HBr, Phenylephrine HCl, and Chlorpheniramine Maleate, as illustrated in Fig 2 a. This decision was based on the achieved resolution, peak shape. Sharpness, elution and sensitivity values observed during the trials. All three API was monitored at 220 nm, as this wavelength demonstrated the highest sensitivity for the compound. Under optimized chromatographic conditions, improved peak shape, Notably, trial results depicted sharp peak shapes and enhanced resolution and maximum extraction, as illustrated in Fig 2 b. Implementation of the gradient elution method resulted in improved resolution, enhanced detection, superior peak, and increased overall efficiency.

ANALYTICAL METHOD VALIDATION-

Linearity –

The standard stock solution of dextromethorphan HBr, phenylephrine HCl, and Chlorpheniramine maleate was created by dissolving dextromethorphan HBr, phenylephrine HCl, and Chlorpheniramine maleate reference standard in diluent. Linearity was demonstrated across the concentration range of 90 to 210 µg/ml, 30 to 70 µg/ml, 12 to 28 µg/ml. [5]

Precision Studies –

Precision studies were conducted through three distinct approaches: intraday precision, interday precision, and repeatability. Intraday Precision involved assessing precision for a single concentration over the course of a single day at various time intervals (Morning, afternoon, evening). Interday Precision was evaluated over multiple days (day-1, day-2, and day-3) for a single concentration. Repeatability studies were carried out by analysing one concentration six times. [11]

Recovery/Accuracy Studies –

Recovery studies were performed by spiking the sample with the standard and then finding out the recovery at solutions of 80%, 100% and 120% were prepared. In this a chunk of standard drug was mixed to the placebo sample and find out recovered amount from sample preparation as % recovery. [7]

Limit of Detection (LOD) and Limit of Quantitation (LOQ) -

For analytical methods characterized by baseline noise, the Limit of Detection (LOD) can be determined using a signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. This ratio is typically expressed as the % concentration of the analyte in the sample. The LOD and LOQ calculation involves injecting three repetitions followed by regression analysis. Subsequently, the standard deviation (SD) is calculated and the slope is utilized to compute the LOD. [9]

$$\text{LOD} = 3.3 \times \text{SD} / \text{Slope}$$

$$\text{LOQ} = 10 \times \text{SD} / \text{Slope}$$

Specificity-

To analyse the specificity of the method different chromatograms was obtained for sample, placebo, blank and standard to ascertain that the method is specific or not or is there any interference from any impurities or excipients. [5]

Robustness –

Robustness studies were performed by injecting working standard preparation for different wavelength, flow rate, different buffer pH and column temperature. Wavelength from +0.2units to -0.2units, Flow rate was changed from +0.2ml/minute to -0.2ml/minute, Buffer pH from+0.2 pH to -0.2pH and column temperature from +2°C to -2°C. [6]

RESULTS AND DISCUSSION-**Linearity–**

Range of the proposed HPLC method, curves was constructed by plotting peak areas obtained for the analyte against their concentrations. A good linear relationship ($r^2 > 0.998$) was observed between the concentration of dextromethorphan HBr, phenylephrine HCl, chlorpheniramine maleate and their corresponding peak areas. The data represented in table no. 2. a, b, c and the calibration curves are presented in fig. 3. a, b, c.

Sr. no.	Conc. (µg/ml)	Mean Area.	Peak Statistical Analysis
1	90.0	35.4967	$y = 0.3962x + 0.2122$ $R^2 = 0.9999$
2	120.0	47.1247	
3	150.0	58.6221	
4	180.0	71.2535	
5	210.0	83.3959	

Table No. 2 a : linearity data- concentration verses area for Dextromethorphan HBr

Sr. no.	Conc. (µg/ml)	Mean Area.	Peak Statistical Analysis
1	30.0	15.2420	$y = 0.5128x + 0.1668$ $R^2 = 0.9997$
2	40.0	20.0151	
3	50.0	25.2340	
4	60.0	30.7869	
5	70.0	35.9110	

Table No. 2 b : linearity data- concentration verses area for Phenylephrine HCl

Sr. no.	Conc. (µg/ml)	Mean Area.	Peak Statistical Analysis
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1	12.0	6.0660	$y = 0.5252x - 0.1402$ $R^2 = 0.9992$
2	16.0	8.0629	
3	20.0	10.2746	
4	24.0	12.6468	
5	28.0	14.6287	

Table No. 2 c : linearity data- concentration verses area for Chlorpheniramine Maleate

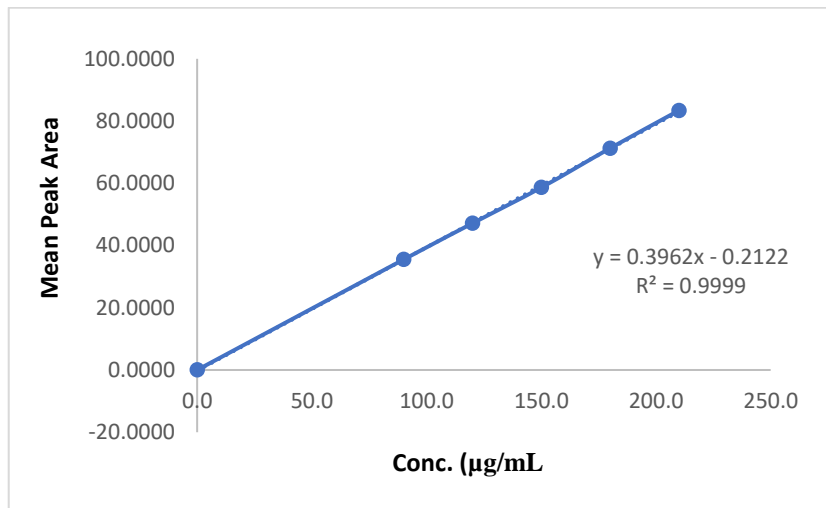


Fig 03. a: Calibration Curve for Dextromethorphan HBr

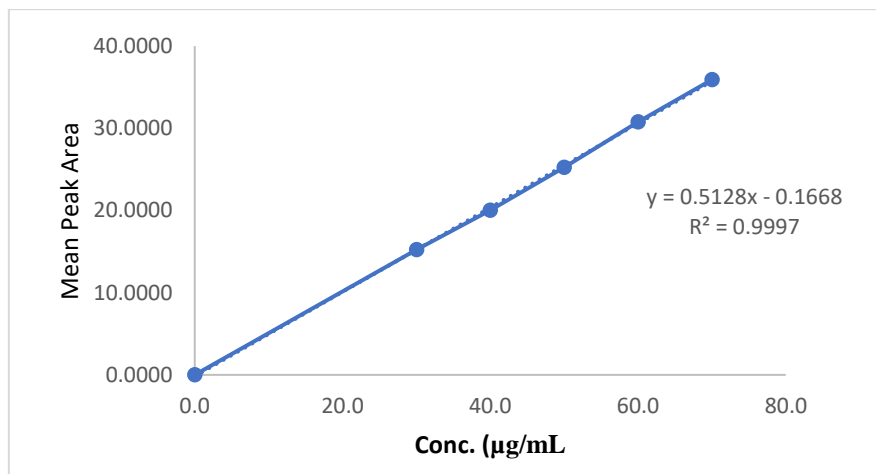


Fig. 03 b: Calibration Curve for Phenylephrine HCl

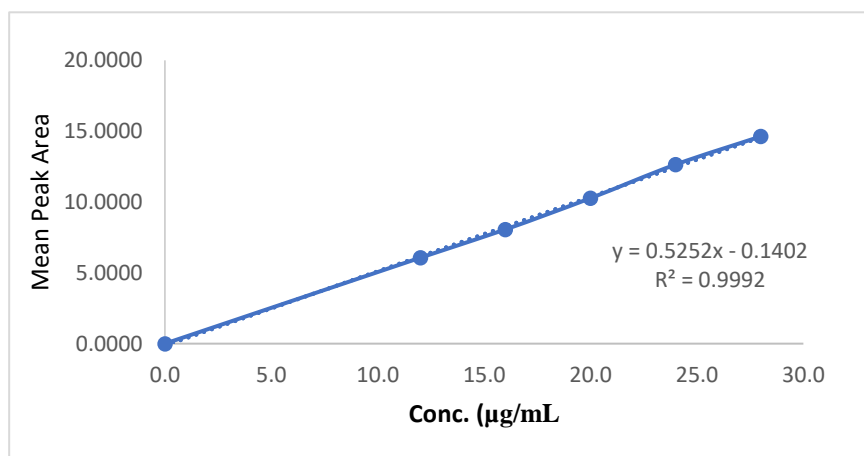


Fig. 03 c: Calibration Curve for Chlorpheniramine Maleate

Precision studies –

Precision is the level of reproducibility of the results as reported between sample analyzed on the same day (intra-day) and samples run on the different days (inter-day). To check intra-day and inter-day variation of method solutions containing 150, 50, 20 µg/ml of Dextromethorphan hydrobromide, Phenylephrine HCl, and Chlorpheniramine maleate, respectively, were subjected to the proposed HPLC method of analysis and the results obtained were noted. The precision of the proposed method i.e. the intra and inter-day variations in the peak areas of the drugs solutions were calculated in terms of percent RSD and the results are presented in table 4 and 5. A statistical evaluation revealed that the relative standard deviation of the drug at linearity level for 6 injections was less than 2.0.

Table 4- Intra-day precision of the proposed method for Dextromethorphan HBr, Phenylephrine HCl, Chlorpheniramine maleate.

Time	Peak Area of Dextromethorphan HBr			Peak Area of Phenylephrine HCl			Peak Area of Chlorpheniramine maleate		
	Morning	Afternoon	Evening	Morning	Afternoon	Evening	Morning	Afternoon	Evening
Avg.	60.6906	60.0814	61.2523	25.4685	25.4907	25.43822	10.8167	10.6466	10.5044 5
SD	0.0894	0.0412	0.1118	0.0574	0.0544	0.0501	0.0539	0.0487	0.0157

% RSD	0.1473	0.0686	0.1825	0.2254	0.2134	0.1968	0.499	0.457	0.150
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Table 5 : Inter- day precision of the proposed method for Dextromethorphan HBr, Phenylephrine HCl, Chlorpheniramine maleate

Days	Peak Area of Dextromethorphan HBr			Peak Area of Phenylephrine HCl			Peak Area of Chlorpheniramine maleate		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Avg.	66.72977	67.89135	86.66228	28.5884	30.30793	31.6284	13.60828	13.84028	14.78398
SD	0.0830	0.0976	0.8497	0.1061	0.1393	0.1131	0.0525	0.0288	0.0275
% RSD	0.1243	0.1437	0.9920	0.3712	0.4597	0.3575	0.3855	0.2080	0.1859

Specificity:

An aqueous solution of dextromethorphan HBr, phenylephrine HCl, and Chlorpheniramine maleate was prepared and injected into the column and the retention time was checked by comparing the response in the blank. No interference was observed at the retention time for the respective drug. The method was found to be precise and specific drug. A typical chromatogram of dextromethorphan HBr, phenylephrine HCl, and chlorpheniramine maleate standard and sample are shown in fig. 03 (a, b, c, d).

Fig 03. a: A chromatogram obtained for the blank

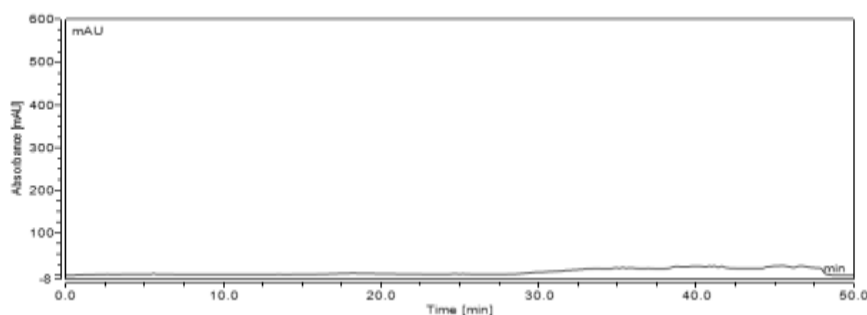


Fig 03. b : A chromatogram obtained for the mixture of dextromethorphan HBr, phenylephrine HCl, and chlorpheniramine maleate (Placebo).

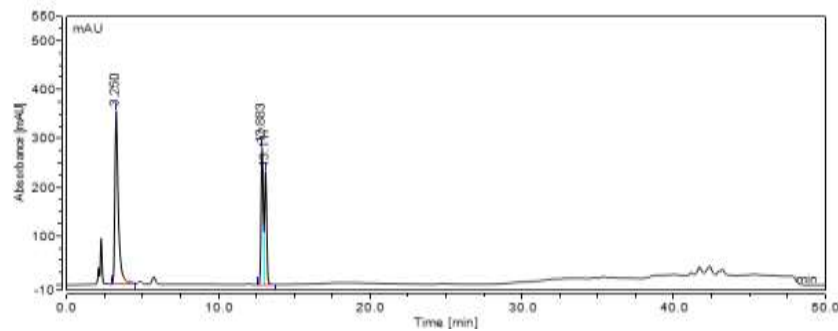


Fig 03. c: A Typical chromatogram obtained for the mixture of dextromethorphan HBr, phenylephrine HCl, and chlorpheniramine maleate (standard).

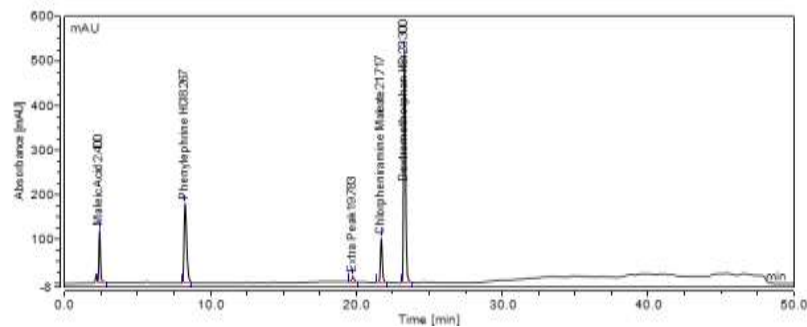
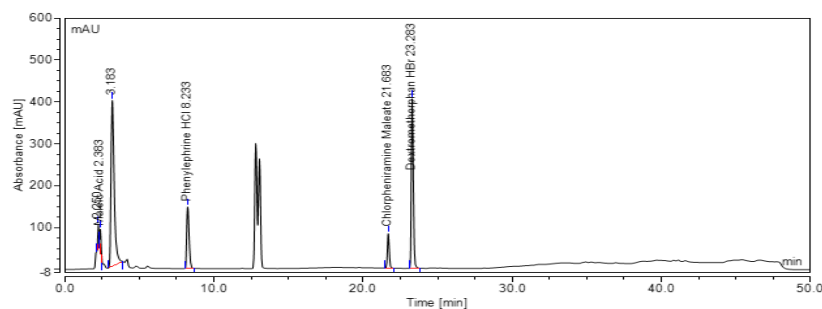


Fig 03. d: A Typical chromatogram obtained for the mixture of dextromethorphan HBr, phenylephrine HCl, and chlorpheniramine maleate (sample).



Accuracy-

Percentage recovery was found to be within limits. The study was done at three different concentration levels 80%, 100% and 120%. for Dextromethorphan HBr, Phenylephrine HCl,

Chlorpheniramine Maleate, respectively. Results obtained from recovery studies are given in table no. 6 with average recovery found out to be known amount of standard was added to the pre analyzed sample and subjected to the proposed HPLC analysis.

Table 6 - Area of sample, Percentage recovery, standard deviation and % R.S.D for Dextromethorphan HBr, Phenylephrine HCl, Chlorpheniramine maleate

API	Test Area	% Recovery	SD	% RSD
Dextromethorphan HBr	50.7967	98.0977	0.0591	0.0035
	66.11573	101.4292	0.1020	0.0103
	76.384	99.3232	0.0625	0.0039
Phenylephrine HCl	20.8792	99.1564	0.0486	0.0024
	26.5122	101.2302	0.0510	0.0026
	32.56953	101.9331	0.0952	0.0089
Chlorpheniramine maleate	8.1536	100.1928	0.0400	0.0016
	10.3308	100.9797	0.4152	0.1708
	12.80627	103.6610	0.0566	0.0031

Limit of detection (LOD) and Limit of quantitation (LOQ):

LOD was found to be 0.011, 0.162, 0.102 and LOQ was found to be 0.034, 0.491, 0.310 for Dextromethorphan HBr, Phenylephrine HCl, and Chlorpheniramine Maleate.

Assay studies-

Assay results are given in Table 7, which shows the area of standard, area of samples, average assay, standard deviation and % R.S. D of assay of Dextromethorphan HBr, Phenylephrine HCl and Chlorpheniramine maleate, respectively.

Table 7- Area of standard, area of samples average assay, standard deviation and % R.S.D of assay

API	Average. % Assay	Standard Deviation	% RSD of Assay
Dextromethorphan HBr	99	0.65629	0.66001

Phenylephrine HCl	100	0.718934	0.71633
Chlorpheniramine maleate	101	0.669116	0.66332

Specificity-

To assess the method's specificity, chromatograms were generated for the blank, placebo, commercial pharmaceutical formulation, standard, and sample as depicted in fig. 03 (a, b, c, d). The observation revealed the absence of any interference from impurities and excipients in the developed method.

Robustness:

The optimized HPLC conditions were slightly modified to evaluate the robustness of the method. Small variations were made in the mobile phase ratio and flow rate. From the results, it was indicated that the selected factors remained unaffected by small variations in these quantities as well as the method was robust even by change in flow rate ± 0.1 mL/min and change in detection wavelength ± 5 nm. The results are shown in Table 8.

Table 8: Results of Robustness Study for dextromethorphan HBr, phenylephrine HCl, and chlorpheniramine maleate.

Parameters	Dextromethorphan HBr			Phenylephrine HCl			Chlorpheniramine Maleate		
	RT	AREA	% RSD	RT	AREA	% RSD	RT	AREA	% RSD
Temp.									
28°C	23.40	66.5242	0.0241	8.40	23.7737	0.0110	21.70	9.9313	0.0513
30°C	23.26	67.3854	0.3223	8.15	24.1997	2.6596	21.58	10.0536	0.1201
32°C	23.10	66.9474	0.9469	7.86	24.9554	0.1203	21.43	9.5593	0.5055
Flow rate									
0.8 ml/min	24.93	83.0775	0.0308	10.08	30.1732	0.0606	23.33	12.7692	0.0304
1.0 ml/min	23.20	68.2500	0.0297	8.08	24.4703	0.0547	21.51	10.2555	0.0595
1.2 ml/min	21.88	56.5472	0.0449	6.76	20.1659	0.0629	20.15	8.3277	0.0511
Wavelength									
218 nm	23.20	67.6019	0.0301	8.10	26.8605	0.5104	21.51	9.6858	0.0497

220 nm	23.20	68.2485	0.0278	8.10	24.4695	0.0522	21.51	10.2541	0.0808
222 nm	23.20	68.2522	0.0306	8.10	21.3675	0.0474	21.51	10.7589	0.0435
pH									
3.0	23.18	66.3704	0.4988	8.06	23.8803	0.0470	21.43	10.0562	0.2518
3.2	23.18	67.0606	0.0355	8.03	23.8302	0.4572	21.51	10.0753	0.0868
3.4	23.18	67.5214	0.0674	8.03	24.0591	0.0506	21.51	10.2146	0.0233

Conclusion-

A review of the literature highlights that RP-HPLC stands out as the most utilized method, outperforming various spectroscopic and chromatographic alternatives in the analysis of these drugs. The existing resources did not yield satisfactory results. However, we failed to achieve satisfactory outcomes for our formulation through the application of the analytical methods outlined in various research papers; hence, the development of a more relevant and accurate method is deemed necessary. The exploration of existing resources has proven to be unsatisfactory in addressing the pertinent issues at hand. Considering this, there arises a compelling need for the development of a more relevant and accurate method to meet the specific requirements of the research. Consequently, the endeavor to create a novel method is fueled by the quest for improved precision, relevance, and overall effectiveness in addressing the objectives of the study.

Conflict of Interest-

Nil

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