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An gradient HPLC-DAD determination of phenylepherine, paracetamol, ambroxol and levocetrizine in pharmaceutical formulation

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The development, validation and application of a simple and reliable gradient high-performance liquid chromatography–diode array detection (HPLC–DAD) procedure for the analysis of a complex mixture containing phenylephrine (PHE), paracetamol (PAR), ambroxol (AMB) and Levocetirizine (LEV) has been carried out . Chromatographic separation of PHE, PAR, AMB and LEV is achieved using a Phenomenex Ultracarb ODS-C18 (4.6×150 mm, 5 µ) column with gradient elution of the mobile phase composed of 10 mM phosphate buffer *p*H 3.3 and acetonitrile. A three step gradient program has been developed with step-1 elution starting with 2% (by volume) acetonitrile which ramped up linearly to 50% in 10 min, in step-2 reverting back to 20% in 5 min and in step-3 ended to achieve initial concentration of 2% in next 5 min thus contributing a total run time of 20 min. Flow rate maintained throughout the experiment is 1 mL/min. The Diode array detector (DAD) is set at 220 nm for quantification of the analytes based on measuring their peak areas. The retention times for PHE, PAR, AMB and LEV are approximately 4.4, 10.1, 14.00 and 17.90 min respectively. The proposed HPLC procedure is statistically validated with respect to linearity, ranges, precision, accuracy, selectivity and robustness. Calibration curves are found to be linear in 50 to 150% of target analyte in formulation with correlation coefficients > 0.9996. The validated HPLC method is applied successfully with good recoveries of analytes from tablet dosage; no interfering peaks were encountered from the inactive ingredients.

Keywords: Phenylephrine, Paracetamol, Ambroxol, Levocetirizine, Gradient, HPLC-DAD, Tablet dosage form.

Phenylephrine chemically is (1R)-1-(3hydroxy-phenyl)-2-(methylamino) ethanol hydrochloride and is used as sympathomimetic (descongestants), Paracetamol is analgesic and antipyretic chemically it is N-(4-hydroxyphenyl) acetamide. Ambroxol [2-amino-3, 5 dibromo-N (trans-4-hydroxy cyclohexyl)] benzyl amine hydrochloride is an expectoration improver and is used in the treatment of respiratory diseases associated with viscid or excessive mucus. Levocetirizine dihydrochloride chemically it is (RS)-2-{4-[(R)-*p*-chloro-á-phenylbenzyl]-1-piperazinyl} ethoxyacetic acid dihydrochloride it is a third generation non-sedative antihistamine, acts by blocking histamine receptors. It is used in the treatment of several allergic reactions, viz., allergic rhinitis, idiopathic urticaria, hay fever etc. Structural formulas of PHE, PAR, AMB and LEV are given in Fig. 1.

Literatures revels number of analytical methods published for PHE, PAR, AMB and LEV alone and with some other drug combinations.

PHE is an official drug in pharmacopoeias such as BP and USP. The BP reports a potentiometric titration with 0.1M ethanolic sodium hydroxide for the assay of PHE, BP also reports a spectrophotometric absorption maximum method for PHE in injections[1]. The USP recommends a volumetric method involving bromometric titration analysis for the assay of PHE moreover USP also

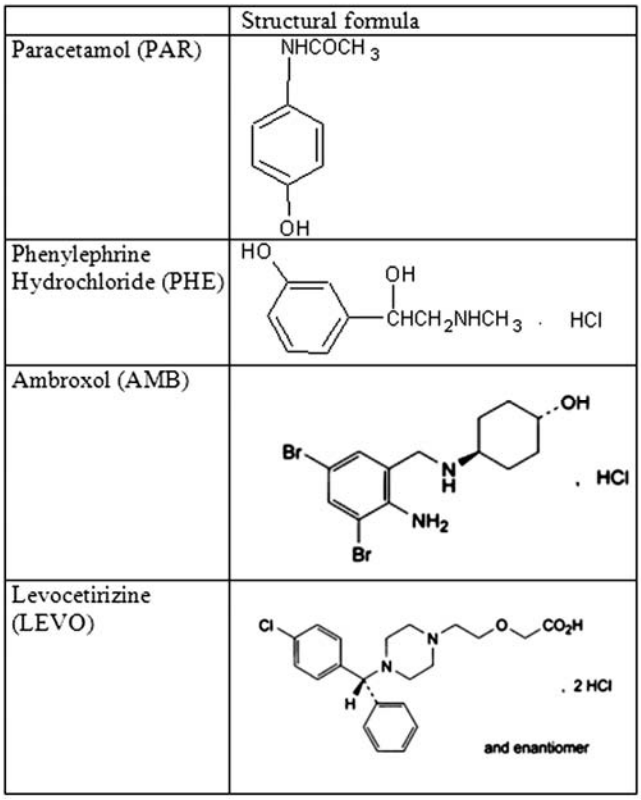

Fig. 1 — The structures of paracetamol (PAR), phenylephrine hydrochloride (PHE), ambroxol (AMB) and levocetirizine (LEVO)

reports several HPLC methods for PHE in various dosage forms[2]. Literature also proposes a wide variety of analytical techniques for PHE, such as HPLC[3], technique making use of electrochemical sensor[4], anodic voltammetry on a modified glassy carbon electrode[5], spectrophotometry[6,7], derivative spectrophotometry[8], chemometric spectrophotometry[9], flow-injection spectrophotometry[10,11], flow injection analysis with chemiluminescence detection[12], capillary electrophoresis[13,14].

Analytical methods for quantification of PAR and its combinations with other analytes or in biological fluids have been reported. PAR has been determined in combination with other drugs using fluorimetry[15], colorimetry[16], UV-spectrophotometry[17], quantitative thin-layer chromatography (TLC)[18], high-performance liquid chromatography (HPLC)[19-25] and gas chromatography (GC)[26] in pharmaceutical dosage form. Ambroxol the third analyte of combination have been reported to be analyzed by HPLC[27,28]. Analytical methods such as spectrometry[29], HPLC-flourimetry[30] and Capillary electrophoresis with flourimetric detection[31].

LEV the other analyte of the multidrug combination has been reported to be quantified by HPLC in plasma[32]. A UV-spectrometry method has been reported for analysis in tablet formulation[33]. A capillary electrophoresis method involved with electro-chemiluminescence detection in human urine have also been reported[34].

This combination of four analytes is indicated in treatment chronic sinusitis, rhinitis, fever, nasal discharge, sore throat and wheezing. All the four analytes present in tablet formulation show variation in chemical structure and hence demonstrate variable polarity and chromatographic behavior making their chromatographic separation difficult. Moreover, the active compounds present in formulation also have variable concentration viz. PHE 5 mg, PAR 500 mg, AMB 60 mg and LEV 2.5 mg such a variation makes the process of simultaneous analysis even more challenging. To our knowledge, the methods described in the literature do not cover the analysis of the combination PHE, PAR, AMB and LEV in pharmaceutical formulations. Therefore, the main objective of this work was to develop a single separation method for analyzing these four analytes which are present in variable concentrations in tablet dosage form.

Within this context, a simple alternative methodology for determination of these drugs in tablets using a gradient chromatographic mode of analysis with total run time of 20 min was proposed. After validation of method for various parameters, the method proved to be successful and was applied to the analysis of commercial products containing these active ingredients.

Experimental Section

Chemicals and Reagents

Working standards of pharmaceutical grade phenylephrine hydrochloride, paracetamol, ambroxol hydrochloride and levocetirizine dihydrochloride were obtained as generous gifts from Leben pharmaceuticals (Akola Maharashtra, India). They were used without further purification. Fixed dose combination tablet Cezlevo cold® tablets (Finecure Pharmaceuticals Limited) containing 5 mg PHE, 500 mg PARA ,60 mg AMB and 2.5 mg LEV was purchased from local market, Yavatmal, Maharashtra, India. All the chemicals were of HPLC grade, purchased from Merck Chemicals, India. Water used was double distilled and filtered through 0.45μm filter.

Instrumentation

The HPLC system consisted of waters series 600E pump quaternary gradient, waters online degasser module a 996 photo-diode array (PDA) detector, a 515 autoinjector ; data were acquired and processed by use of EMPOWER software (all equipments from Waters, Milford). The chromatographic separations were carried out on a Phenomenex Ultracarb C-18 column (150 mm × 4.5mm i.d., particle size 5 μm) with gradient conditions.

Preparation of standard stock and sample solution

Sample preparation was done in acetonitrile taking accurately weighed quantity of PHE, PAR, AMB and LEV transferred to 25 mL volumetric flasks separately to give standard stock solution of 50 μg/mL of PHE, 5000 μg/mL PAR, 600 μg/mL AMB and 25 μg/mL LEV.

For preparation of sample solution of tablets twenty tablets (Cezlevo cold® tablets) were weighed and powdered finely. Tablet powder equivalent to 5 mg PHE, 500 mg PARA, 60 mg AMB and 2.5 mg LEV was transferred to a 100 mL volumetric flask and dissolved in 20 mL of acetonitrile and sonicated for 15 min, the volume was further made up to the mark. Resultant was filtered through 0.45 micron membrane filter. The solution was further diluted to obtain resultant concentration of 5 μg/mL of PHE,

500µg/mL of PARA, 60 µg/mL of AMB and 2.5 µg/mL of LEV this mixture was subjected to HPLC analysis in developed chromatographic conditions.

Chromatographic conditions

The mixture of four analytes possessed a degree of variation in chromatographic behaviour due to structural differences; hence the separation needed a gradient mode rather than an isocratic mode for successful separation. The process of separation was achieved using Phenomenex Ultracrab ODS-C18 (4.6×150 mm, 5 µ) column with gradient elution of the mobile phase comprising of 10 mM phosphate buffer adjusted to *p*H 3.3 with orthophosphoric acid and acetonitrile. Effective separation was achieved by a three step gradient program with step-1 elution starting with 2% (by volume) acetonitrile and 98 % of phosphate buffer, which ramped up linearly to 50% in 10 min, in step-2 the acetonitrile concentration reverting back to 20% in a time interval of 5 min and at last in step-3 ended to achieve initial concentration of 2% acetonitrile and 98 % phosphate buffer in next 5 min thus contributing a total run time of 20 min. The mobile phase was pumped at a flow rate of 1 mL/min and the eluants were monitored at 220 nm. The 10 mM phosphate buffer was filtered through 0.45 micron membrane filter and degassed before use. The injection volume was 20 µL and all analyses were performed at ambient temperature.

Results and Discussion

Method development and optimization of chromatographic conditions

The complexity of analytical mixture demanded a variation in polarity at every stage so as to provide sufficient resolution with acceptable peak symmetry in a reasonable analysis time. A gradient liquid chromatographic method involved with diode array detection was developed to provide a suitable procedure for the routine quality control analysis of mixtures of PHE, PAR, AMB and LEV in tablet dosage form.

To achieve this goal, several experiments were carried out to optimize both the stationary and mobile phases. For optimization of the stationary phase variety of reversed-phase columns such as Technochrome-C8 (4.6 × 150 mm, 3.5 µ), Grace-C18 (4.6 × 250 mm, 5µ), Nucleosil C18 (4.6 × 250 mm, 5µ) and Phenomenex Ultracarb ODS-C18 (4.6 × 150 mm, 5 µ) were tested. The Phenomenex Ultracarb ODS-C18 column provided the best resolution between the analytes in a complex mixture and hence was chosen as a column of choice for this study.

For optimal separation of analytes parameters such as ideal mobile phase and their proportions at optimum *p*H were exhaustively studied so as to achieve a reasonable degree of separation of analytes. Several binary or ternary eluants were tested using different proportions of solvent, such as acetonitrile, methanol, water and buffer at different *p*H conditions. However, satisfactory results were achieved by using 10 mM phosphate buffer of *p*H 3.3 adjusted with ortho-phosphoric acid and acetonitrile. Methanol was experimented as an organic modifier due to its cost benefits but the mixture comprised of compounds of varied polarity with compound like LEV being the most nonpolar showing a strong affinity to reverse-phase stationary phase hence acetonitrile was selected as a choice of organic modifier for the method.

The chromatographic separation was a three step gradient program operated at 1 mL/min with step-1 elution starting at 98% of polar phase (phosphate buffer) and 2% (by volume) of acetonitrile ramping up linearly to 50% of each in a time interval of 10 min. The first step needed a start with high amount of polar phase decreasing gradually with simultaneous increase of acetonitrile so as to separate the structurally close compounds PHE and PAR. Step-2 followed immediately after step -1 with further changes involved with decrease of acetonitrile back to 20% in next 5 min and the method finally ended in step-3 to achieve initial concentration of 98% phosphate buffer and 2% acetonitrile in next 5 min so as achieve ideal conditions for next chromatographic run. The eluants were monitored at 220 nm a common wavelength showing a significant absorbance of all four analytes. Quantification was achieved based on peak area measurement. Figure 2 shows a typical chromatogram for standard mixture for the separation of the four analytes PHE, PAR, AMB and LEV eluted at retention times 4.4, 10.1, 14.0 and 17.9 min, respectively with resolution (Rs) of greater than 1.5 between all the peaks demonstrating a good degree of separation between adjacent peaks (A value of 1.5 for Rs implies a complete separation of any two consecutive peaks)[35]. Table 3 shows analytical parameters such as retention time, asymmetry, tailing and theoretical plates obtained for the optimal chromatographic conditions.

Method validation

Selectivity and Linearity

Method selectivity was assessed by the peak purity test (comparison between analyte peak and auto threshold in the purity plot) using diode array detector.

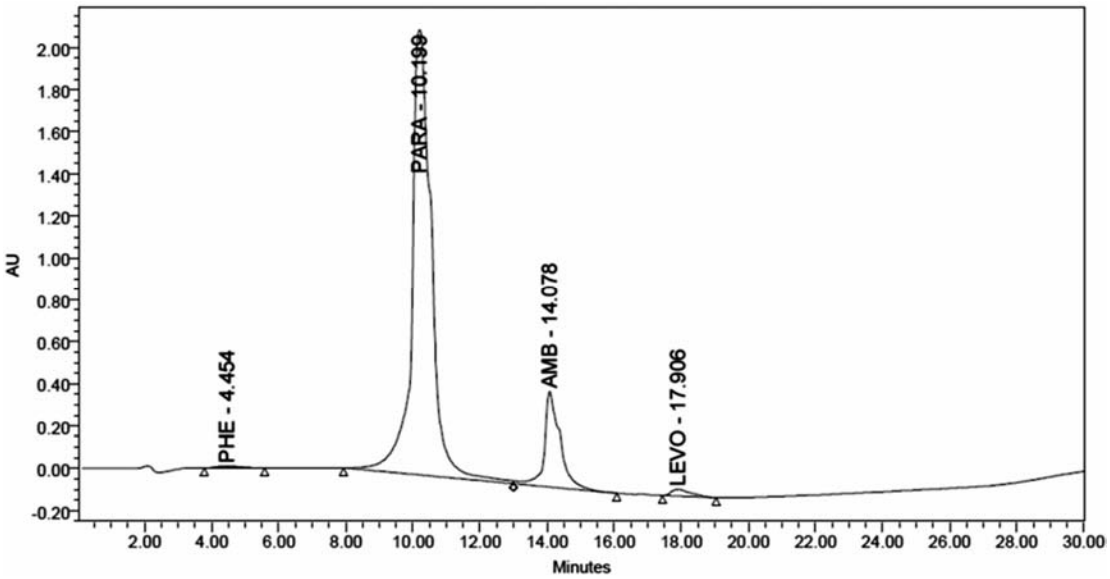

Fig. 2 — HPLC chromatogram obtained during simultaneous determination of PHE, PAR, AMB and LEVO.

The analyte chromatographic peak was not found to be attributable to more than one component indicating the method to be selective[36].

For linearity, an external method was used for the simultaneous determination of four ingredients. Five concentrations were chosen ranging from 50 to 150% at five levels of the target analyte concentrations in formulations. So the concentrations were for PHE 2.5 to 7.5 µg/mL PAR 250 to 750 µg/mL, AMB 30 to 90 µg/mL and LEV 1.25 to 3.75 µg/mL. All the solutions were prepared in acetonitrile. Each point was analyzed three times (n=3). Each concentration of standard mixture solutions was injected in triplicate and the mean value of peak area was taken for the calibration curve. Calibration graph was obtained by plotting peak area versus concentration of standard drugs. The linear regression equations for PHE, PAR, AMB and LEV were found to be. The regression coefficient values (R^2) were found to be 0.998, 0.997, 0.988 and 0.994 respectively indicating an acceptable degree of linearity.

Specificity

The specificity of method was accessed from the chromatogram where complete separation of PHE, PAR, AMB and LEV was achieved and against potential interferences in the presence of placebo. The peaks obtained were sharp and well separated at the baseline also excipients from formulation were not interfering with assay no interferences were detected at retention times of PHE, PAR, AMB and LEV in sample solution proving the method to be specific.

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision is determined through the estimate of the relative standard deviation (RSD) values. The precision in the validation of this optimized method was performed at two levels: repeatability and intermediate precision.

Repeatability (n=6) in sample area was carried out for 100.0% of the test concentration. In the present case, concentrations at 5, 500, 60, 30 and 2.5 µg/mL for each PHE, PAR, AMB and LEV respectively were used. Intermediate precision (n=6) was performed on different days. All results presented acceptable precision values (not exceeding 5.00%) as shown in Table 1.

Accuracy

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as % recovery determined by standard addition method. Accuracy was assessed by spiking the active ingredients into the placebo at different concentrations 80%, 100%, and 120% each of the labelled claim and injected in developed chromatographic conditions in triplicate. The recovery data for accuracy studies is shown in Table 2.

System Suitability Parameters

For system suitability parameters, seven replicate injections of mixed standard solution were injected

Table 1 — Precision studies OF PHE, PAR, AMB and LEV

Concentration µg/mL	Mean measured concentration±%RSD	
	Repeatability (n=6)	Intermediate precesion (n=3)
	PHE	
2.5	2.45 ± 2.5	2.40 ± 2.0
5	4.95 ± 2.7	5.0 ± 1.5
7.5	7.40 ± 2.2	7.5 ± 1.2
	PAR	
250	251.80 ± 2.2	252.50 ± 1.5
500	502.00 ± 3.5	501 ± 1.2
750	755 ± 2.2	753.80 ± 2.3
	AMB	
30	30.5 ± 1.5	29.80 ± 2.3
60	59.5 ± 2.5	58.80 ± 2.2
90	88.5 ± 2.5	89.10 ± 2.3
	LEV	
1.25	1.20 ± 1.6	1.22 ± 1.5
2.5	2.55 ± 1.5	2.45 ± 2.6
3.75	3.70 ± 2.8	3.65 ± 2.3

Table 2 — Accuracy studies of PHE, PAR, AMB and LEV

Recovery level	Std. added to placebo	Amount added (mg)	Mean recovery (mg) ± %RSD (n=3)	Mean % Recovery
50%	PHE	2.5	2.45 ± 2.4	98.00
	PAR	250	250.50 ± 2.6	100.20
	AMB	30	29.90 ± 2.2	99.66
	LEV	1.25	1.22 ± 1.6	97.60
100%	PHE	5.0	4.90 ± 2.5	98.00
	PAR	500	502.50 ± 2.8	100.50
	AMB	60	60.50 ± 1.4	100.83
	LEV	2.5	2.40 ± 2.2	96.00
150%	PHE	7.5	7.3± 1.6	97.33
	PAR	750	752.50 ± 1.5	100.33
	AMB	90	90.60 ± 1.3	100.66
	LEV	3.75	3.65 ± 1.4	97.33

and parameters such as the retention time, asymmetry factor, tailing factor and theoretical plates of the peaks were calculated. The results are shown in Table 3.

Robustness studies

Robustness of the developed method was evaluated by deliberate minor modifications in chromatographic conditions.

The parameters included variation of flow rate, *p*H and detecting wavelength. Robustness studies were carried out using a mixture at concentration levels 5 µg/mL PHE, 500 µg/mL PAR, 60 µg/mL for AMB and 2.5 µg/mL LEV. The system suitability parameters considered for deliberate changes were %RSD of peak areas, mean tailing factor and mean retention time.

Table 3 — System suitability studies of PHE, PAR, AMB and LEV

Std. Sol.	Parameters (* mean values) n=7			
	RT*	Asymmetry*	Tailing*	Theoretical Plates*
PHE	4.4	1.10	1.20	4822
PAR	10.1	1.62	1.70	18145
AMB	14.0	1.94	1.40	9680
LEV	17.9	1.85	1.95	25210

Table 4 — Analysis of marketed formulation by proposed method

Commercial formulation	Ingredients	Labeled amount (mg)	Amount found (mg)	Found %
Cezlevo cold tab®	PHE	5	4.75	95.00
	PAR	500	505.60	101.12
	AMB	60	61.20	102.00
	LEV	2.5	2.55	102.00

Analysis of formulation

The proposed HPLC method was applied to simultaneous determination of PHE, PAR, AMB and LIV in Cezlevo cold®. The quantitative results of these assays are summarized in Table 4. Satisfactory results were obtained for each compound in good agreement with labeled claims. No interferences of excipients were seen in chromatogram.

Conclusion

In this study, a validated simple and reliable HPLC–DAD method has been described for the assay of multi drug combination having PHE, PAR, AMB and LEV which pose a challenge to analytical process by showing variability of chromatographic behaviour and concentration in formulation. To our present knowledge, no attempts have yet been made to assay this four drug mixture by any analytical methodology. The four analytes (phenylephrine, paracetamol, ambroxol and levocetirizine) were successfully resolved and quantified using a RP-C18 column in a run time of 20 min; the developed method made use of the diode-array detector as a tool for peak purity confirmation. The developed method was validated and was found to be simple, precise, accurate and sensitive. The method was successfully applied to assay of marketed tablet formulation. The proposed method is specific as the excipients present in the dosage form provide no interference in the determination of the active constituents. Hence the method can be recommended for the routine quality control of the studied drugs, either in bulk form or in combination in some other formulations.

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Hydrochloride, Paracetamol, Ambroxol hydrochloride and Levocetirizine dihydrochloride.

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