Arabian Journal of Chemistry (2015) **8**, 591–598

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Development and validation of a novel RP-HPLC method for simultaneous determination of paracetamol, phenylephrine hydrochloride, caffeine, cetirizine and nimesulide in tablet formulation

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Received 6 May 2013; accepted 19 September 2013
Available online 9 October 2013

KEYWORDS

Phenylephrine;
Paracetamol;
Caffeine;
Cetirizine;
Nimesulide;
HPLC–DAD

Abstract The present work describes development and validation of a high-performance liquid chromatography–diode array detection (HPLC–DAD) procedure for the analysis of phenylephrine hydrochloride (PHE), paracetamol (PAR), caffeine anhydrous (CAF), cetirizine Dihydrochloride (CET), nimesulide (NIM) in pharmaceutical mixture. Effective chromatographic separation of PHE, PAR, CAF, CET and NIM was achieved using a Kinetex-C18 (4.6 mm, 150 mm, 5 mm) column with gradient elution of the mobile phase composed of 10 mM phosphate buffer (pH 3.3) and acetonitrile. The elution was a three step gradient elution program step-1 started initially with 2% (by volume) acetonitrile and 98% phosphate buffer (pH 3.3) for first 2 min. In step-2 acetonitrile concentration changed linearly to 20% up to 12 min the analysis was concluded by step-3 changing acetonitrile to 2% up to 20 min. The proposed HPLC method was statistically validated with respect to linearity, ranges, precision, accuracy, selectivity and robustness. Calibration curves were linear in the ranges of 5–100, 100–1000 and 10–200 mg/mL for PHE, PAR, CAF, CET and NIM

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2013.09.040

respectively, with correlation coefficients >0.9996. The HPLC method was applied to tablet dosage form in which the analytes were successfully quantified with good recovery values with no interfering peaks from the excipients.

1. Introduction

Phenylephrine (PHE) chemically is (1R)-1-(3hydroxy-phenyl)-2-(methylamino) ethanol hydrochloride and is used as sympathomimetic (descongestants). Paracetamol (PAR) is analgesic and antipyretic chemically it is N-(4-hydroxyphenyl) acetamide. Caffeine (CAF) chemically is (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione and acts as a central nervous system stimulant. Cetirizine Dihydrochloride (CET) provides prompt relief of itchy watery eyes, runny nose, sneezing, itching of the nose or throat due to respiratory allergies chemically it is (±)-[2-[4-[(4-chlorophenyl)phenylmethyl]-1-piperazinyl]ethoxy]acetic acid dihydrochloride. Nimesulide (NIM) is a selective COX-2 inhibitor which provides analgesic & antipyretic effect chemically it is N-(4-Nitro-2-phenoxyphenyl) methanesulfonamide. Structural formulas of PHE, PAR, CAF, CET and NIM are given in Fig. 1.

The mixture of five drugs is recommended to relieve symptoms such as nasal and sinus congestion, allergic symptoms of the nose or throat due to upper respiratory tract allergies and sinus pain associated with headache. The multidrug mixture is also used as an adjunct with antibacterials in sinusitis, tonsillitis, and otitis media.

The tablet contains a variable amount of all ingredients due to their recommended pharmacological dose; the tablet contains 10 mg of phenylephrine hydrochloride, 325 mg of paracetamol, 25 mg caffeine, 5 mg cetirizine and 100 mg of nimesulide. This variable amount of ingredients in such a

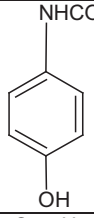
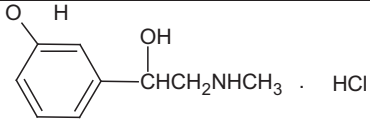
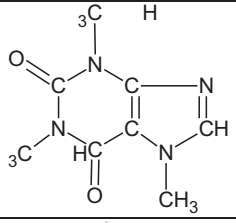
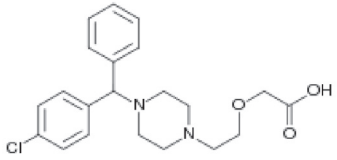
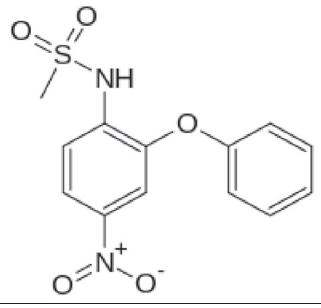
	Structural formula
Paracetamol (PARA)	
Phenylephrine Hydrochloride (PHE)	
Caffeine (CAF)	
Cetirizine Dihydrochloride	
Nimesulide	

Figure 1 The structures of Paracetamol (PAR), Phenylephrine hydrochloride (PHE), Caffeine (CAF), Cetirizine (CET) and Nimesulide (NIM).

multi-drug formulation makes the process of routine analysis difficult. Moreover, the active compounds have very different polarity and, therefore chromatographic behavior. So far no single HPLC method is reported to determine the mentioned ingredients quantitatively in this combination.

The literature reveals a number of analytical methods published for PHE, PAR, CAF, CET and NIM with some other drug combinations.

Methods for paracetamol and its combinations in pharmaceuticals or in biological fluids have been reported. Paracetamol has been determined in combination with other drugs using titrimetry (British Pharmacopoeia, 1998; Pharmacopoeia et al., 1997), voltammetry (Saeed and Reyhaneh-Sadat, 2011), fluorimetry (Hossein and Yahya, 2011), colorimetry (Shihana et al., 2010), UV-spectrophotometry (Ghulam et al., 2011), quantitative thin-layer chromatography (TLC) (Atul and Afsar, 2008), high-performance liquid chromatography (HPLC) (El-Kommos et al., 2012; Godse et al., 2009; Franeta et al., 2002; Issa et al., 2012; Gopinath et al., 2007; Olmo et al., 2005) and gas chromatography (GC) (Belal et al., 2009) in pharmaceutical formulations.

An HPLC method for phenylepherine in combination with Chlorpheniramine Maleate has been reported (Mukesh et al., 2010).

Caffeine has been analyzed in combination with some other active agents by a variety of analytical methods such as spectrophotometry (Kuldeep et al., 2011), HPLC (Viswanath et al., 2011) and HPTLC (Misra et al., 2009).

Cetirizine has been reported for analytical methods such as HPLC for estimations in formulations and plasma (Maithani et al., 2010; Nagaralli et al., 2003), spectrophotometry (Bhatia et al., 2008), Capillary zone electrophoresis (Azhagvuel and Sekar, 2007) a stability indicating assay method is also reported (Hadada et al., 2009; Khan et al., 2011). Nimesulide the other analyte of multidrug combination has been quantified by analytical methods such as HPTLC (Patravale et al., 2001), UV spectrophotometry (Altinöz and Dursun, 2000), differential pulse voltammetric (Wang et al., 2006), FT-NIR spectroscopy (Ajayakumar et al., 2012) and HPLC (Tzanavaras and Themelis, 2007).

To the best of our knowledge, the methods described in the literature do not cover the analysis of five analytes PHE, PAR, CAF, CET and NIM in a pharmaceutical mixture in the form of tablet formulation. Therefore, the main objective of this work was to develop a single separation method for quantifying these five analytes which are present in variable concentrations in tablet dosage form.

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

2. Experimental

2.1. Chemicals and reagents

Working standards of pharmaceutical grade phenylephrine hydrochloride, paracetamol, caffeine anhydrous, cetirizine dihydrochloride and nimesulide were obtained as generous gifts from Leben pharmaceuticals (Akola Maharashtra, India). They were used without further purification. Fixed dose combination tablet Ness cold plus® (One Ness Pharmaceuticals Ltd) containing 10 mg phenylephrine hydrochloride, 325 mg Paracetamol, 25 mg caffeine, 5 cetirizine and 100 mg nimesulide 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 0.45 μm filter.

2.2. Instrumentation

The HPLC system consisted of waters series 600E pump quaternary gradient, waters online degasser module a 996 photodiode array (PDA) detector, a 515 autosampler; data were acquired and processed by making use of EMPOWER software (all equipments from Waters, Milford). The chromatographic separations were carried out on a reverse phase Kinetex-C18 column (150 × 4.5 mm i.d., particle size 5 μ, core shell technology).

2.3. Preparation of standard stock and sample solution

2.3.1. Preparation of standard stock solution

Preliminarily sample preparation was done in acetonitrile taking accurately weighed quantity of 10 mg of PHE, 325 mg of PAR, 25 mg of CAF, 5 mg of CET and 100 mg of NIM transferred to 100 ml volumetric flasks separately to give stock solutions of 100 μg/ml of PHE, 3250 μg/ml of PAR, 250 μg/ml of CAF, 50 μg/ml of CET and 1000 μg/ml of NIM.

2.3.2. Preparation of mixed standard solution

A mixed standard solution was prepared from these stock solutions by transferring 10 mL of each of the stock solution to a 100 mL volumetric flask and diluting with acetonitrile to get a solution of 10, 325, 25, 5 and 100 μg/ml of PHE, PAR, CAF, CET and NIM respectively.

2.3.3. Preparation of sample solution of tablet

For preparation of sample solution of pharmaceutical mixture twenty tablets (Ness cold plus® Tab) were weighed and powdered finely. Tablet powder equivalent to 10 mg of PHE, 325 mg of PAR, 25 mg of CAF, 5 mg of CET and 100 mg of NIM was transferred to a 100 ml volumetric flask and dissolved in acetonitrile up to the mark. The solution was ultrasonicated for 15 min and filtered through 0.45 μ membrane filter. The solutions were further diluted to obtain resultant concentration of 10 μg/ml of PHE, 325 μg/ml of PAR, 25 μg/ml of CAF, 5 μg/ml of CET and 100 μg/ml of NIM the resultant mixture was subjected to HPLC analysis in developed chromatographic conditions.

2.4. Chromatographic Conditions

Initial trials were carried by an isocratic mode of analysis using the mixture of phosphate buffer and organic phase acetonitrile and methanol. Looking to the variability in polarities trials were initiated on reverse phase Kinetex C-18 column (150 × 4.5 mm i.d., particle size 5 μ) made by core shell

technology from Phenomenex. Experiments concluded lack of resolution of a complex mixture of five drugs using the isocratic approach of analysis. The gradient mode was opted comprising buffer and acetonitrile as organic phase. Mobile phase composed of 10 mM phosphate buffer (pH 3.3) and acetonitrile. The elution was a three step gradient elution program with flow of 1 ml/min throughout the method, step-1 started initially with 2% (by volume) acetonitrile and 98% phosphate buffer for 2 min, acetonitrile concentration changed linearly to 80% in next 10 min followed by final step-3 reverting acetonitrile concentration back to 2% and phosphate buffer 98% in last 8 min thus concluding the method in total run time of 20 min, a tabular representation of developed gradient program is given in Table 1. The eluants were monitored at 230 nm. The mobile phase was filtered through 0.45 μ membrane filter and degassed before use. The injection volume was 20 μl and all analyses were performed at ambient temperature. Figs. 2 and 3 show the chromatogram for standard mixture and spectrum index plot obtained through the optimized variables in accordance with the features described above.

3. Results and discussion

3.1. Method development and optimization of chromatographic conditions

The development of the method was based on the experience obtained from the HPLC method previously developed for the analysis of mixture of analytes comprising phenylephrine, paracetamol, caffeine and chlorpheniramine maleate (Dewani et al., 2012) (30) by authors. Experiments previously suggest use of C-18 stationary phases of (150 × 4.5 mm i.d., particle size 5 μm) hence for the study a reverse phase Phenomenex Kinetex-C18 column made by core shell technology was utilized. For the separation of all the five analytes in mixture the composition and pH of mobile phase were varied. Parameters such as mobile phase composition of buffer at different pH values 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 pH conditions. Initially isocratic mode of separation was experimented and was found insufficient to resolve the mixture with good peak characters hence gradient mode was selected so as to achieve separation of analytes with good peak characters. The optimized gradient program was varied by changing the organic phase in programed steps. Initially a four step gradient program gave good separation of all the five analytes with total run time of 25 min, in order to reduce the total run time it was optimized further. The final optimized gradient program was a three step gradient program having total run time of 20 min thus reducing the total run time by 5 min. The mean retention time of five analytes was PHE 1.8, PAR 9.81, CAF 11.70, CET 14.17 and NIM 16.35 min respectively. Peak identification was done by injecting individual analyte in developed chromatographic conditions. A value of 1.5 for resolution implies a complete separation of any two consecutive peaks (British Pharmacopoeia, 2010). Resolution was calculated between the adjacent peaks of PHE, PAR, CAF, CET and NIM which was > 1.5 indicating an adequate degree of resolution.

Table 1 Description of gradient program.

Step no.	Phosphate buffer (%)	Acetonitrile (%)	Time (min)
Step 1	98	2	2
Step 2	20	80	12
Step 3	98	2	20

3.2. Method validation

3.2.1. 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. The analyte chromatographic peak was not found to be attributable to more than one component indicating the method to be selective (Conference Harmonisation (ICH), 1995).

For linearity, an external method was used for the simultaneous determination of five ingredients. Five concentrations were chosen ranging from 50% to 150% of the target analyte concentrations in formulations. So the linearity dilution concentrations were PHE 5–15 μg/ml, PAR 162.5–487.5 μg/ml, CAF 12.5–37.5 μg/ml, CET 2.5–7.5 μg/ml and NIM 50–150 μg/ml. All the solutions were prepared by diluting in acetonitrile. 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, CAF, CET and NIM were found to be $y = 1994x + 3388$, $y = 51{,}421x + 25{,}293$, $y = 9641x + 16{,}376$, $y = 18{,}743x - 30{,}719$ and $y = 39{,}548x + 22{,}485$ respectively. The regression coefficient values (R^2) were found to be 0.998, 0.997, 0.996, 0.999 and 0.996 respectively indicating an acceptable degree of linearity.

3.2.2. Specificity

The specificity of the method was accessed from the chromatogram where complete separation of PHE, PAR, CAF, CET and NIM was achieved and against potential interferences in the presence of placebo (diluents i.e., acetonitrile). 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, CAF, CET and NIM in sample solution proving the method to be specific. A chromatogram for placebo studies is shown in Fig. 4.

3.2.3. 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. Precision studies were carried by carrying inter-day and intra-day studies. The precision studies were done by injecting the prepared standard solution at three concentration levels (50%, 80% and 150%) in triplicate every day up to three consecutive days for inter-day studies. Intra-day studies were done by injecting the standards at three different times on same day. %RSD values were measured the low value of RSD (%) showed that the method is precise within the acceptance limit of ±2%. The intra- and inter-day variability or precision data are given in Table 2. The results indicated good precision of the developed method.

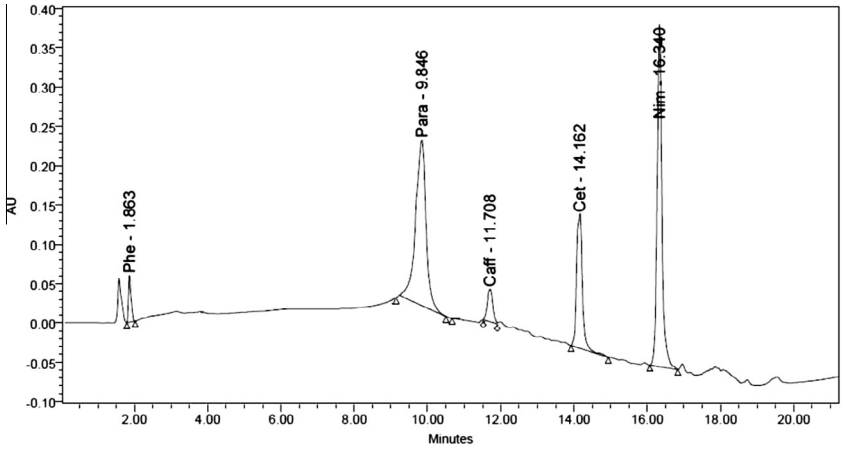


Figure 2 HPLC chromatogram obtained during simultaneous separation of PHE, PAR, CAF, CET and NIM. Chromatographic conditions: Kinetex C-18 (150 × 4.5 mm i.d., particle size 5 μ) (Phenomenex); mobile phase gradient elution of three steps, phosphate buffer 10 mM, pH adjusted to 3.3 with ortho-phosphoric acid, and acetonitrile with step 1. (98:2) v/v for 2 min. step 2. (20:80) up to 12 min. and step 3 again achieving initial concentration of 98:2 up to 20 min. flow rate of 1.0 ml/min; and UV detection at 230 nm.

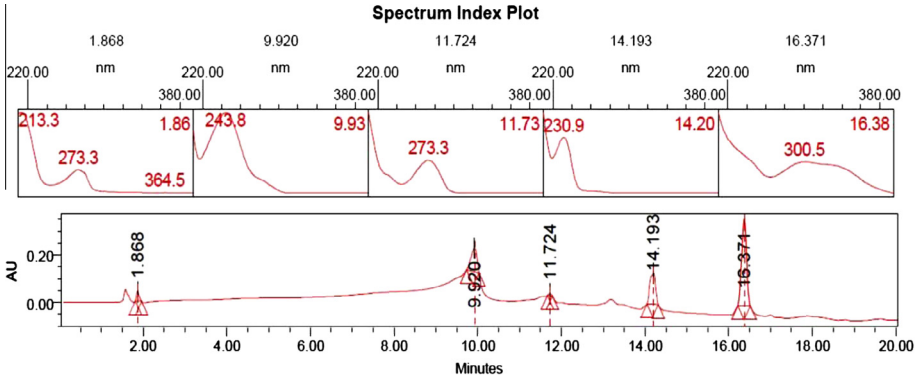


Figure 3 Spectrum Index plot for simultaneous HPLC estimation of PHE, PAR, CAF, CET and NIM in pharmaceutical mixture.

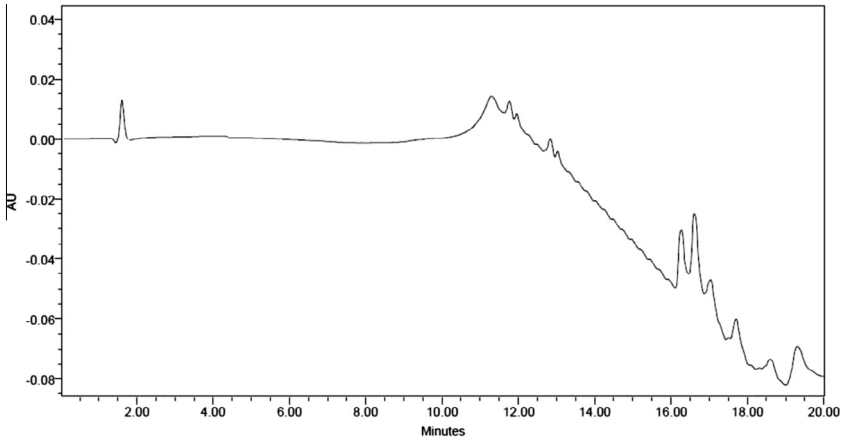


Figure 4 Chromatogram showing placebo runs in developed chromatographic conditions.

3.2.4. Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value, which is accepted reference value, and the value found. Accuracy studies were done by the standard addition method. Accuracy is expressed as % recovery of the standard spiked to previously analyzed test sample of tablet. The active ingredients were spiked in previously analyzed tablet powder sample at different concentration levels viz. 80%, 100%, and 120% each of the labeled claim and injected in developed chromatographic conditions in triplicate.

Table 2 Precision data of intra-day and inter-day assay ($n = 6$).

Concentration µg/ml	Measured mean concentration ± %RSD	
	Inter-day precision ($n = 6$)	Intra-day precision ($n = 6$)
PHE		
5	4.9 ± 1.2	4.95 ± 1.5
10	10.10 ± 2.2	10.15 ± 1.2
15	15.20 ± 1.2	15.10 ± 2.3
PAR		
162.5	163.2 ± 1.8	164.8 ± 1.5
325	327.5 ± 1.3	328.8 ± 2.2
487.5	493 ± 1.5	492.6 ± 1.2
CAF		
12.5	12.40 ± 1.3	12.60 ± 1.8
25	25.40 ± 1.7	25.20 ± 2.2
37.5	37.90 ± 1.2	37.20 ± 1.8
CET		
2.5	2.4 ± 1.2	2.5 ± 1.5
5	4.80 ± 1.2	4.90 ± 1.6
7.5	7.7 ± 1.8	7.6 ± 1.4
NIM		
50	50.5 ± 1.6	50.9 ± 2.3
100	102.3 ± 2.8	101.8 ± 2.2
150	152.3 ± 1.3	153.3 ± 2.1

Table 3 Recovery data for accuracy studies.

Recovery level	Std. added to placebo	Amount added (mg)	Mean recovery (mg) ± %RSD ($n = 3$)	Mean % Recovery
80%	PHE	8	8.1 ± 1.4	101.25
	PAR	260	263.6 ± 2.2	101.38
	CAF	20.0	19.6 ± 2.2	98.00
	CET	4.0	4.1 ± 2.6	102.5
	NIM	80	82.50 ± 1.8	103.12
100%	PHE	15	15.30 ± 1.5	102.00
	PAR	325	328.50 ± 1.8	101.07
	CAF	25	24.8 ± 1.4	99.22
	CET	5	5.15 ± 1.9	103.00
	NIM	100	98.3 ± 2.2	98.30
120%	PHE	18	17.70 ± 1.6	98.33
	PAR	390	394.60 ± 1.5	101.17
	CAF	30	30.6 ± 1.2	102.00
	CET	6	6.05 ± 1.1	100.83
	NIM	120	123.20 ± 2.2	102.66

The percentage recoveries were calculated from the slope and *Y*-intercept of the calibration curve. The recovery data for accuracy studies are shown in Table 3.

3.2.5. System suitability parameters

System suitability tests are an integral part of the analytical method it is used to verify adequacy of the resolution and reproducibility of system. For study of system suitability parameter, seven replicate injections of mixed standard (100% level of labeled claim) solution were injected and parameters such as peak area, retention time, asymmetry factor and theoretical plates of the peaks were calculated. The results are shown in Table 4.

3.2.6. Robustness studies

The robustness of a method is the ability to remain unaffected by small changes in chromatographic parameters. The experimental conditions were purposely altered and the chromatographic resolution of PHE, PAR, CAF, CET and NIM was assessed. The chromatographic parameters included variation of flow rate second deliberate change was made by change in pH and third was deliberate change in detection wavelength. To study the effect of flow rate on system suitability parameters ±10% change on either side of actual flow rate was made i.e., from 1.0 to 1.1 mL/min and 0.9 mL/min, while other conditions were held constant. For experiment to study the effect of pH on system suitability parameters change in pH of ±0.1 units on either side of actual pH of buffer was made i.e., from 3.3 to 3.4 and 3.2 while other chromatographic conditions were kept constant. For variation of detecting wavelength change in detecting wavelength of ±5 nm was made and system suitability parameters were recorded. All the robustness studies were carried using a mix standard having resultant concentration of 10 µg/ml for PHE, 325 µg/ml for

Table 4 System suitability data.

Std. Sol.	Parameters (* mean values) $n = 7 \pm$ SD			
	RT*(min)	Peak area	Asymmetry*	Theoretical plates*
PHE	1.8 ± 0.09	203999.3 ± 815.56	1.18 ± 0.02	657575 ± 3468.73
PAR	9.81 ± 0.12	5173470 ± 10541.9	1.6 ± 0.02	1531592 ± 20135.13
CAF	11.70 ± 0.01	986707 ± 4423.13	0.92 ± 0.01	315331 ± 4366.86
CET	14.17 ± 0.01	191474 ± 10481.3	1.02 ± 0.01	102295 ± 2213.37
NIM	16.35 ± 0.01	3986067 ± 4497.61	0.92 ± 0.005	584520 ± 2973.26

* Results are mean values ($n = 7$) ± SD

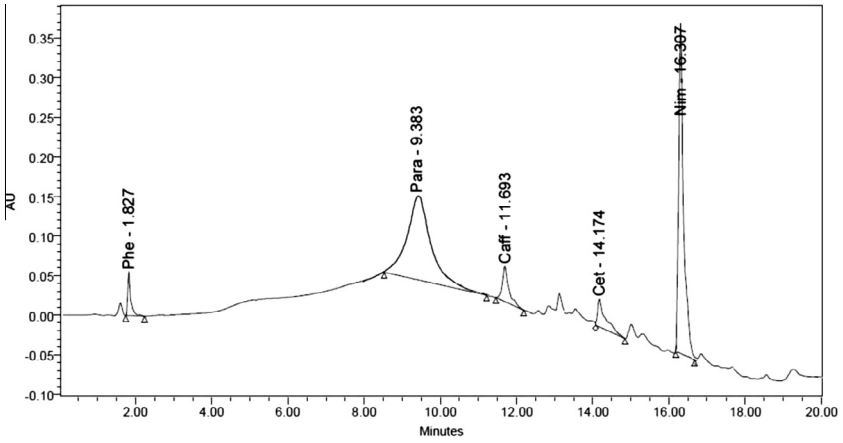


Figure 5 Chromatogram for marketed preparation.

Table 5 Results for assay of marketed formulation.

Commercial formulation	Ingredients	Labeled amount (mg)	Amount found (mg)	Found %
Ness cold plus®	PHE	15	14.95	99.67
	PAR	325	328.30	101.01
	CAF	25	25.80	103.20
	CET	5	5.20	104.00
	NIM	100	103.20	103.20

PAR, 25 μg/ml for CAF, 5 μg/ml for CET and 100 μg/ml of NIM. The system suitability parameters considered for deliberate changes were %RSD of peak areas, mean tailing factor and mean retention time.

3.2.7. Analysis of formulation

The developed method was successfully applied to analyze PHE, PAR, CAF, CET and NIM in marketed tablet formulation. The amounts recovered were expressed as a percentage of the label claim. Analysis of marketed tablets Ness cold plus® (One Ness Pharmaceuticals Ltd) was carried out in developed chromatographic conditions. No interferences of excipients were observed in analysis, a representative chromatogram for analysis of tablet formulation is shown in Fig 5. The mean percentage recovery of drug contents of tablets obtained by the proposed method was noted. The percentage recovery found was 99.67% for PHE, 101.01% for PAR, 103.20%, CAF 104% CET and 103.2% NIM. The results are given in Table 5.

4. Conclusion

In this study, a validated simple and reliable HPLC–DAD procedure was described for the assay of a complex multi drug combination consisting of PHE, PAR, CAF, CET and NIM which is indicated for the treatment of allergic symptoms of the nose or throat due to upper respiratory tract allergies associated with headache. To our present knowledge, no attempts have yet been made to assay this multidrug mixture by any analytical methodology. All the five analytes (PHE, PAR, CAF, CET and NIM) were successfully resolved and quantified using a Reverse phase Phenomenex Kinetex-C18 column in a relatively short run time with the last analyte eluting at 16.3 min the gradient program contributed total run time of 20 min. Reliability was guaranteed as validation experiments proved that the HPLC method is linear in the proposed working range as well as accurate, precise and specific. The good recovery percentage of tablet forms suggests that the excipients

have no interference in the determination. The RSD (%) was also less than 2 showing a high degree of precision of the method. The proposed method was also found to be robust with respect to flow rate, pH of mobile phase and detecting wavelength hence it can be recommended for the routine quality control of the studied drugs, either in bulk form or in their combination formulated in some other dosage form.

Acknowledgments

The authors are grateful to Leben Labs (Akola, Nagpur) for providing gift samples of Paracetamol, Caffeine, Phenylephrine Hydrochloride, Cetirizine dihydrochloride and Nimesulide. Authors are also thankful to management and Dr. A.V.Chandewar, principal of P.W.College of pharmacy, Yavatmal for providing timely support for the research work.

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