Development and Validation of Rapid, Timesaving, and Cost-effective UHPLC Method for Simultaneous Quantification of Cinnarizine, its Five Specified Impurities, Two Degradation Products and Two Antioxidants

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Abstract: This article explains the developed and validated rapid, timesaving, and cost-effective UHPLC method for simultaneous quantification of cinnarizine, its five specified impurities (Impurity-A to E), two degradation products (cinnamyl piperazine and benzhydrol), and two antioxidants (methylparaben and propylparaben). Furthermore, when coupled with a mass spectrometer, the proposed method provides additional advantages for confirmation of results and correct identification based on molecular weight. All analytes were eluted within 15 minutes on an ACQUITY, UPLC, BEH C18 (150 mm x 2.1 mm, 1.7 µm) column at 40.0°C by using two mobile phases containing different compositions of 10 mM ammonium acetate, acetonitrile, and acetic acid in gradient elution mode. The linearity curves of cinnarizine, its impurities, and degradation products showed good results in a correlation coefficient of 0.999 with a lower detection limit (0.1125 μg/mL) and quantification limit (0.1875 μg/mL) at 230 nm. A forced degradation study on spiked and unspiked solutions proved their specificity with improvements and their significance. This proposed method involves a lower flow rate (0.35 mL/min.) with a shorter run time, which provides faster analysis, reduces wastage, reduces the cost, and specifies the greener advantages. The outcome of the validation as per ICH guidelines proved that the proposed UHPLC method is accurate, precise, and timesaving for simultaneous quantification of all analytes in active pharmaceutical ingredient, tablets, capsules, and oral suspension of cinnarizine.

Keywords: Cinnarizine; Specified Impurities; Degradation Products; Antioxidants; Simultaneous Quantification.

Introduction
Cinnarizine, chemically known as (E)-1-(Diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine, is an anti-allergic, anti-emetic, calcium channel blocker, anti-H1-receptor antagonist, antihistamine, antimuscarinic, and anti-vasoconstrictor compound. In the treatment of nausea, vomiting, peripheral vertigo, central vertigo, chemotherapy, and inner ear disorders, cinnarizine is used as the best choice and is administered orally in the form of a tablet, capsule, or oral suspension. The presence of traces of starting materials and different byproducts of the reaction are treated as the main impurities in the final pure form of active pharmaceutical ingredient (API). In addition, the reactions like hydrolysis, oxidation, and thermal degradation of API and drug products generate different specified and unspecified impurities. Cinnarizine has five specified impurities, named...
Impurity A to E. A literature review shows that acidic hydrolysis of cinnarizine yields two degradation products named "cinnamyl piperazine" and "benzhydrol." In the liquid form of an oral suspension formulation of cinnarizine, methylparaben and propylparaben are added as antioxidants. The molecular structures of cinnarizine, its five specified impurities, two degradation products, and two antioxidants are shown in Fig. 1.

In previous papers, we described a validated reverse phase-High Performance Liquid Chromatography (RP-HPLC) method for achieving the same goal with a run time of 38.0 minutes and a flow rate of 1.0 mL/min. When compared to HPLC, the RP-UHPLC (Reverse Phase Ultra High-Performance Liquid Chromatography) method is especially beneficial for rapid analysis, time savings, cost-effectiveness, significantly increased productivity, improved separation, improved sensitivity, reduced solvent consumption, and shorter run durations without compromising efficiency. Because of the increased efficiency of smaller particle sizes in columns and a smaller internal diameter of tubing, ultra-high-performance liquid chromatography (UHPLC) techniques are specially constructed to withstand and work at higher pressures and hence allow for a significant reduction in flow rate. Decreased solvent consumption lowers the cost of each sample analyzed and curtails the waste generated during analysis, which is today’s individual responsibility toward the social responsibility goal of waste management. The UHPLC method, in coupling with mass spectrometer with an electrospray ionization (ESI-MS), allows significant advantages in rapidity, selectivity, sensitivity, and accuracy for rapid screening of desired as well as undesired compounds.

According to the findings of the literature review, there is no published and reported single UHPLC method available to estimate related substances of cinnarizine and its assay from API,
tablets, and capsules, as well as to include two antioxidants in an oral suspension formulation. For quantification of cinnarizine as a single content, with other drugs, or in the presence of limited impurities in pharmaceutical and biological samples, there are different reported techniques, which include potentiometry \(^{1-3}\), spectrophotometry \(^9,10\), voltammetry \(^{11}\), capillary electrophoresis \(^{12}\), and chromatographic methods \(^{13-25}\). Additional information about introduction part, different synthesis routes of cinnarizine and review literature are provided in supplementary information (Table S1, S2, and Fig. S1).

In this article, a rapid and timesaving UHPLC method has been developed and validated for the simultaneous determination of cinnarizine, its five specified impurities (Impurity-A to E), two degradation products (cinnamyl piperazine and benzhydrol), and two antioxidants (methylparaben and propylparaben). In conjugation with an electrospray ionization mass spectrometer, the proposed UHPLC method is helpful for correct identification and confirmation of quantitative results based on observed molecular weight. The results of the validation as per ICH (The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) guidelines \(^ {26,27}\) demonstrated the different capabilities of the proposed UHPLC method.

**Experimental work**

**Chemicals and reagents**
The supplementary information section (2.1 and Table S3) contains information on all chemicals, reagents, standards, placebos, and samples.

**Instruments and software**
All chromatographic analysis was performed on the Waters, Acquity Ultra High-Performance Liquid Chromatography (UHPLC) system equipped with Photodiode-Array (PDA) detector and Quadrupole Dalton (QDa) mass spectrometer (MS) detector with an electrospray ionization (ESI) source.

**Preparation of solutions**
The supplementary information section (2.3) contains all the detailed information and procedures on the preparation of solutions.

**Method development**
The core goal of this research work was to develop a rapid, time-saving, and cost-effective RP-UHPLC method for simultaneous separation and quantification of all ten analytes in different pharmaceutical dosage forms. During the method development, a number of diverse combinations of different concentrations of aqueous buffer and organic phase on all selected UHPLC columns were evaluated based on information in the reported methods, the solubility of all analytes, and the characteristics of the analytical columns. The peak responses and system suitability parameters were investigated and evaluated at different detection wavelengths with different injection volumes of spiked standard solution. At the end, all optimized parameters are fine-tuned cumulatively for improved outcome and to finalize the UHPLC chromatographic conditions and mass spectrometer parameters. Spiked standard solutions with and without two antioxidants and acid hydrolyzed spiked sample standard solutions were used in method development activity.

**Method robustness**
Robustness is the ability of an analytical method to remain unaffected and be found to be reliable even when performed with a slight change in defined parameters. The robustness of the proposed method was evaluated to prove that the variation in chromatographic parameters was not impacting the results. The robust nature of the developed UHPLC method was examined and proved with the minimum and maximum impact of six different parameters mentioned in the proposed chromatographic conditions.

**Forced degradation study**
To demonstrate the specificity of the proposed methods, force degradation (FD) studies have been performed on the cinnarizine API, unspiked API solution (dissolved API without added impurities), and spiked API solution (dissolved API with added impurities and degradation
products). These API and dissolved API (with and without impurities) were stressed by acid hydrolysis (HCl), base hydrolysis (NaOH), oxidative degradation (H₂O₂), and heat stress (70°C), and these were intended to generate and separate all potential degradants from specified impurities and the cinnarizine peak. The study's proposed outcome was to develop a method that would be a single, selective, sensitive, and practical method for determining all specified and unspecified cinnarizine impurities. The purpose of the FD study evaluation of the spiking cinnarizine API solution was to examine the degradation pattern and confirm the co-elution of any degradants with specified impurities and an API peak.

Analytical method validation
The proposed and developed UHPLC method with finalized chromatographic condition parameters was validated as per ICH guidelines. Validation of the method in terms of specificity, accuracy (n = 3), precision (system repeatability, analysis repeatability, and intermediate precision), limit of detection (LOD), limit of quantification (LOQ), linearity, range, solution stability, filtration study, relative response factor, and system suitability test. The additional information with respect to method validation parameters and methodology is listed in the supplementary information (Table S4).

Optimization of the mobile phases
The most efficient way to improve the separation of multiple analytes is to adjust the selectivity because these parameters are purely related to the stationary phase and the mobile phase that are used in the experiment. Hence, the objective of this optimization was to figure out which constituents are required in mobile phases and how to use them in gradient elution mode to achieve the best outcome. To separate all ten analyte peaks, different ratios of ammonium acetate in water, acetonitrile, and acetic acid were tested as mobile phases. The diverse ratios containing acetic acid (0.05, 0.1, and 0.2%), ammonium acetate (10 and 20 mM), and acetonitrile were tried with a constant flow rate in gradient elution mode along with acetonitrile and methanol as diluents on the octadecylsilane (C18) stationary phase column individually and separately. With slight differences, excellent peak separation was observed with all the diverse sets of mobile phases containing these three constituents (reagent and solvents) in gradient elution mode along with methanol as a diluent on the C18 column. However, mobile phases containing mobile phase-A (0.05% acetic acid in 10 mM ammonium acetate/acetonitrile, 90/10, v/v) and mobile phase-B (0.05% acetic acid in 10 mM ammonium acetate/acetonitrile, 10/90, v/v) offer the best separation and most stable baseline compared to other ratios and strengths.
of solutions. The chromatogram of a solution prepared in methanol as a diluent shows higher peak response, higher peak height, higher plate counts, and a slightly controlled tailing factor than the chromatogram of a solution prepared in acetonitrile as a diluent.

**Optimization of detection wavelength**

Use of the PDA detector extends its usefulness by providing spectra of analyte peaks for correct peak identification and confirmation of peak purity or absence of co-elution. The recorded PDA spectra of different injected solutions in the range of 190 - 400 nm was extracted and checked at different wavelengths to finalize a single detection wavelength for all ten analytes based on the stable baseline and higher peak response. The wavelength less than 220 nm shows the baseline on a negative scale, while the wavelength above 240 nm shows the baseline on a higher positive scale. The detection wavelength of 230 nm provided a better outcome compared to other wavelengths based on a better positive scale baseline near-zero baseline. Based on peak area responses, the detection wavelength of 240 nm was not a suitable wavelength for the quantification purpose of three analytes (Impurity-A, benzhydrol, and Impurity-E), while the detection wavelength of 220 nm is an excellent suitable wavelength for the quantification purpose of these three analytes. Based on peak area responses of all ten analytes, the detection wavelength of 220 nm was providing excellent peak area responses with a baseline on a negative scale, while the detection wavelength of 230 nm was offering moderate peak area responses with a baseline on a positive scale. The detection wavelength of 230 nm was finalized based on the baseline on the positive scale and higher peak responses for the main peak (cinnarizine) and impurity-B. The overlay chromatogram of spiked standard solution extracted at different detection wavelengths (220, 230, and 240 nm) is shown in Fig. 2.

**Optimization of column chemistry**

The primary goal of this optimization step was to adjust a selected set of factors in such a way that, cumulatively, they would provide a targeted outcome. Hence, to do that, it is important to pick suitable columns that are the perfect combination of length, internal diameter, and particle size. The spiked standard solution was used to evaluate the suitable length of column (50 mm, 100 mm, and 150 mm) with a 2.1 mm internal diameter and 1.7-1.8 µm particle sizes. Diverse UHPLC columns with octadecysilane (C18) stationary phase (Table 1 lists the columns) were tested and screened in gradient elution mode with a different mobile phase. The selection of

![Figure 2. The overlay chromatogram of peak responses of all ten analytes at detection wavelength 220 nm (Black), 230 nm (Red), and 240 nm (Blue)](image_url)
Table 1. List of columns with octadecylsilane (C18) stationary phase selected for optimization of column chemistry

| Make (Item No.) | Column Type  | Length x I.D. mm | Particle Size µm | Pore Size Å | Carbon Load % | pH Range |
|----------------|--------------|------------------|-----------------|-------------|---------------|----------|
| Waters (WT186002350) | UPLC BEH C18 | 50 x 2.1mm | 1.7 | 130 | 18% | 1 - 12 |
| Waters (WT186001132) | UPLC HSS T3 C18 | 100 x 2.1mm | 1.8 | 100 | 11% | 2 - 8 |
| Waters (WT186005298) | UPLC CSH C18 | 150 x 2.1mm | 1.7 | 130 | 15% | 1 - 11 |
| Waters (WT186003540) | UPLC HSS T3 C18 | 150 x 2.1mm | 1.8 | 100 | 11% | 2 - 8 |
| Waters (WT186003376) | UPLC RP Shield C18 | 150 x 2.1mm | 1.7 | 130 | 17% | 2 - 11 |
| Waters (WT186002353) | UPLC BEH C18 | 150 x 2.1mm | 1.7 | 130 | 18% | 1 - 12 |

These columns was evaluated against factors like minimum pore size, maximum carbon load, and a wide pH range. The outcome of the C18 columns was judged on the peak separation and peak parameters like peak shape, height, and responses of all analytes. The chromatogram on the UPLC column with BEH C18, 50 mm x 2.1mm, 1.7 µm shows very fast separation (less than 6 minutes) of the desired analytes. It shows too early elution of the first three peaks with broad shapes, a higher tailing factor, and a maximum resolution of up to 4.0. Higher peak responses of solvent peaks may be impacted on the first eluted peak with merging, fronting of the peak, and separation with minimum resolution. The chromatogram on UPLC column HSS T3 C18, 100 mm x 2.1mm, 1.8 µm shows rapid separation (less than 9 minutes) of the desired analytes. However, it was unable to separate the degradants observed in the acid hydrolyzed sample solution. The best outcomes with respect to peak detection and peak separation were seen on two 150 mm columns. Only these two UPLC C18 columns, named UPLC RP-Shield C18 (150 mm, 2.1 mm, 1.7 µm) and UPLC BEH C18 (150 mm, 2.1 mm, 1.7 µm), provided significantly improved peak separation and hence were chosen for further optimization. The chromatograms of spiked standard solutions evaluated on 50 mm, 100 mm, and 150 mm columns and acid hydrolyzed spiked standard solutions on 150 mm columns are presented in Fig. 3.

Optimization of suitable injection volume

Peak responses are directly proportional to injection volume, but injection volumes also influence peak sharpness, tailing factor, peak shape, peak fronting, and peak height most of the time. Hence, by using optimized parameters, different injection volumes of spiked standard solution from 1.0 to 5.0 µL were injected and evaluated for the best output regarding sharpness, shape, height, tailing, and theoretical plates for all desired analyte peaks. The 100% standard solution (0.375 mg/mL) was evaluated with 2.0 µL on UHPLC equipped with PDA and a TUV detector. Finally, based on the evaluation of maximum and minimum peak responses on
Figure 3. The chromatograms of spiked standard solution (A) on 50 mm column, (B) on 100 mm column, (C) on 150 mm column and (D) acid hydrolyzed spiked standard solution on 150 mm column.
different detectors and output regarding peak height, tailing factor, and theoretical plates for all desired analyte peaks, a 2.0 µL injection volume was found suitable. The chromatogram of a solution with a 5.0 µL injection volume, by using optimized mobile phases and two shortlisted columns, did not offer enough good output regarding peak shape and tailing factor to most of the desired analyte peaks. The overlay chromatogram of spiked standard solutions with 2.0µL injection volume at LOD, at LOQ, and at 2LOQ level is presented in supplementary information (Fig. S2).

**Optimization of gradient with constant flow rate**

Based on the observed chromatographic separation with system suitability parameters and column backpressure and considering the increment in backpressure after the number of injections on the same column, a 0.35 mL/min flow rate offers the best results with an altered initial gradient of mobile phases. The fine-tuning with slight alteration in the initially selected gradient mode of mobile phases was selected in such a way that the first eluted peak of cinnamyl piperazine was eluted earlier but after the void volume. It also shows an improved separation between the cinnarizine peak and impurity-B peak, reduced total run time, and fast elution of low-polarity compounds with improved detection efficiency. The impact of the fine-tuned gradient mode of mobile phases with a 0.35 mL/min flow rate was studied on both optimized columns (BEH C18 and RP Shield C18). The cumulative positive impact was seen in the chromatogram of the BEH C18 column with rapid separation, better resolution, improved tailing factor, and decreased column backpressure. Under the same conditions, the chromatogram from the RP Shield C18 column shows rapid separation and better resolution with a higher tailing factor for four impurity peaks out of ten analytes.

**Final optimized instrumental and analytical conditions**

Based on the evaluation of optimized parameters, all chromatographic analysis was performed using a Waters, Acquity UPLC, H-CLASS system with a PDA detector and a quadrupole Dalton (QDa) mass detector with an electrospray ionization source. The finalized conditions consisted of the Acquity UPLC BEH C18 (stationary phase: Octadecylsilane), 2.1 x 150 mm, 1.7 µm particles (Waters, USA) maintained at a 40°C column temperature. Mobile phase-A (10 mM ammonium acetate in water/acetonitrile/acetic acid, 90/10/0.05, v/v/v) and mobile phase-B (10 mM ammonium acetate in water/acetonitrile/acetic acid, 10/90/0.05, v/v/v) were pumped at 0.35 mL/min in gradient programme mode. The gradient programme was performed as 80-30% of A in 0-5 min, 30-27% of A in 5-8 min, 27-15% of A in 8-9 min, 15-10% of A in 9-10 min, 10-10% of A in 10-12 min, 10-5% of A in 12-16 min, 5-80% of A in 16-17 min, and finally 80-80% of A in 17-20 min. for equilibration. The autosampler was maintained at an ambient temperature (e.g., 22°C) and the injection volume was 2.0 µL. For the chromatogram, the PDA spectra needs to be extracted at a 230 nm detection wavelength. The proposed UHLC method was also evaluated for the mass spectrometer parameters with the mentioned parameters. The peak purity results observed in PDA spectra were confirmed by peak purity results in mass spectra. The parameters of the mass spectrometer were finalized as follows. The \( m/z \) was extracted in both positive and negative ESI modes at 450°C as the probe temperature, with a capillary voltage for positive and negative modes of 0.8 kV. The MS scan in positive mode was set to scan the \( m/z \) from 100.00 to 1000.00. While in Negative mode, scan the \( m/z \) from 100.00 to 600.00. The sampling rate was 10 points/seconds, the gain value was 1.0, with 10V as the cone voltage. All optimized UHPLC chromatographic conditions and mass spectrometer parameters are summarized in supplementary information (Table S5). The representative chromatogram of the spiked oral suspension sample solution observed by the UPLC-PDA-ESI-MS method is represented in supplementary information (Fig. S3).

**Method robustness**

The outcome of the method robustness study proved that the minor variation in chromatographic
condition parameters was not impacting the results. The robustness of the developed method was demonstrated using six different parameters, including (a) buffer concentration (±2 mM), (b) flow rate (±0.05 mL/min), (c) column temperature (±5.0°C), (d) change in initial gradient of mobile phase B (±5%), (e) change in initial slope (±1.0 min.), and (f) organic modifier (acetonitrile) concentration in mobile phase-B (±50 mL/liter). Each generated chromatogram in the robustness study was checked, evaluated, and confirmed for all critical parameters such as (i) impact on resolution in between cinnarizine and impurity-B peak, (ii) impact on USP plate count for cinnamyl piperazine and cinnarizine peak, (iii) maximum USP tailing for cinnamyl piperazine and cinnarizine peak, (iv) % RSD of five replicates of standard solution for peak area of cinnarizine, and (v) recoveries of all ten peaks (impurities and degradants at LOQ, and 10LOQ level while API and antioxidants at 100% level). All observed results met the pre-defined minimum requirements of system suitability criteria. This indicated that the developed method has good selectivity and sensitivity and can be used in routine analysis without any problems. Individually and separately generated chromatograms with 8 mM buffer, 0.30 mL/min flow rate, column temperature (35.0°C), initial gradient (75/25), and gradient slope value of 6 min. demonstrated a lower value of resolution between cinnarizine and impurity-B peaks as well as lower USP plate counts for the cinnarizine peak compared to the nominal conditions parameters. When compared to the nominal condition parameters, the chromatograms with 12 mM buffer and higher column temperature (45.0°C) showed increased resolution between cinnarizine and impurity-B peaks and increased USP plate counts for cinnarizine peak. The recoveries of all impurities and degradation products at the LOQ level were in the range of 90 - 110%, while for impurities and degradation products at the (10 x LOQ) level and API and antioxidants at the 100% level, all recoveries were in the range of 98 - 102%. All observed results met the pre-defined minimum requirements of system suitability criteria. These observations reveal that even the changes in the execution of analysis with the mentioned parameters have a slight impact on system suitability parameters due to retention time shifting, but no impact on final quantification results. The overlay chromatograms of spiked standard solution studied with variation in buffer, flow rate, column temperature, initial gradient, initial slope, and percent of acetonitrile in mobile phase-B (MP-B) are showed in Fig. 4 and 5. For detailed observed results of all critical points evaluated in the robustness study, see Table S6 in supplementary information.

**Forced degradation study**

The force degradation studies were conducted on the cinnarizine API (solid, without dissolving) and the cinnarizine solution (liquid, by dissolving the content). The stressed and unstressed cinnarizine solutions as unspiked (without added impurities) and spiked (with added impurities and degradation products) were demonstrated to show the specificity of the proposed methods. All generated degradants in acid hydrolysis, base hydrolysis, oxidative degradation, and heat stress conditions were well separated from the mentioned specified impurities, degradation products, and cinnarizine peak. The total outcome of this study revealed that the developed UHPLC method is a single, selective, sensitive, and suitable method for the quantification of all specified and unspecified impurities of cinnarizine. The results of the force degradation study of spiked sample solution supported and confirmed the degradation pattern and degradants observed in the unspiked stressed solution, as well as the fact that no degradant co-eluted with the specified impurity and cinnarizine peak. The cinnarizine API, cinnarizine unspiked solutions, and cinnarizine spiked solutions were treated with HCl (0.1, 1, and 10 M), NaOH (0.1, 1, and 5 M) and H₂O₂ (1, 3, and 30%) and stored at 40°C in a water bath for 12 hours. In heat stressed conditions, the cinnarizine API was exposed at 70°C in an oven for 12 hours. While in the case of the heat stressed condition of the unspiked and spiked solutions, both flasks were stressed at 40°C in a water bath for 12 hours.

The final solutions of the all-stressed samples...
and solutions were prepared and analyzed by the proposed UHPLC method and the UHPLC-PDA-ESI-MS method. The total outcome of force degradation with HCl (0.1 and 1 M), NaOH (0.1 and 1 M) and H₂O₂ (1%) was not that helpful in generating the degradants and total impurities of more than 0.2%, including existing impurities. However, the outcome of the force degradation of cinnarizine solutions with 10 M HCl and 30% H₂O₂ showed that dissolved cinnarizine was sensitive to acid hydrolysis and oxidative degradation. The concentrations of cinnarizine solution stressed with 10 M HCl and 30% H₂O₂ were low as compared to the concentration in the unstressed solution. However, the concentration in the stressed solution with 5 M NaOH hydrolysis and heat stress conditions remained unchanged as compared to the added concentrations. The outcome of acidic degradation with 10 M HCl was proved to be that within 12 hours, two of the mentioned degradation products, cinnamyl piperazine and benzhydrol, were generated in the acidic hydrolysis of cinnarizine, and total degradation was up to 4.5%. Due to the high
The concentration of solvent in oxidative degradation, within 12 hours cinnarizine was degraded up to 3.5%, after 4 days the same solution showed 36% degradation, and after 7 days the same solution showed 90% degradation. The major degradant in all chromatograms was observed at RRT_0.90 and at m/z 385.4. The chromatograms of solutions were stressed with a 30% \( \text{H}_2\text{O}_2 \) solution, which confirmed the absence of secondary degradation. The cinnarizine molecular ion appeared at m/z 369.2 in both spiked and unspiked solutions, while one major degradant was identified at RRT_0.9 and the same degradant appeared at m/z 385.4 in mass spectral data. The molecular weight difference between both was 16 m/z and it revealed that there was an addition of one oxygen to the cinnarizine molecule. The mass spectrum revealed that the degradant at RRT_0.9 with m/z 385.4 shows further degradants at m/z 117.1, 167.1, and 267.2. The degradant observed at 267.2 suggests that the one oxygen (m/z: 16) was attached to one of the carbon or nitrogen of the piperazine ring. The peak purity for cinnarizine and impurities was evaluated by using PDA data.
and mass spectral data. Based on peak purity results, it was confirmed that all eluted peaks in the force degradation study were pure and there was no coelution of peaks. The detailed outcome with chromatograms generated in the force degradation study as well as different reactions explaining the formation of possible degradants in acid hydrolysis and oxidation degradation are presented in section 3.3 with Fig. S4 to S17 and Table S7 in the supplementary information.

**Analytical method validation**

Analytical method validation was carried out according to the guidelines for method validation by the International Conference on Harmonization (ICH). It was performed to estimate specificity, accuracy, precision, limit of detection, limit of quantification, linearity, range, stability of solutions, filtration study, relative response factor, carry-over test, and system suitability test.

**Specificity**

The specificity of the proposed method was proved by analyzing the solutions containing (i) dilution solvent as blank, (ii) individual placebo solutions of the tablet, capsule, and oral suspension formulations, (iii) system suitability test solution, (iv) 100% standard solution, (v) individual impurity solution of five specified impurities and two degradation products, (vi) individual solution of both antioxidants, and (vii) a spiked and unspiked sample solution of drug substance and three drug formulations. The peak purity was checked and calculated by the UPLC-PDA scan using “Empower” software. The purity angle values were observed to be lower than the purity threshold values for cinnarizine, five specified impurities, two degradation products and two antioxidants. These results confirmed that there was no coelution of peaks with each other and that the peak of the cinnarizine and its impurities and degradation products were pure. The peak purity was also confirmed with the help of data obtained from the mass spectral. In the chromatogram of a blank matrix, small blank peaks were occasionally noticed between 6.5 and 8.5 minutes of retention time when analytical grade solvent was utilized instead of LC-MS grade solvent. However, the magnitude of these blank peaks was very negligible, and they were separated from the mentioned peak in that region. There were no placebo peaks found. The overlay chromatogram of the blank, tablet placebo, capsule placebo, oral suspension placebo, and spiked standard solution is shown in the supplementary information (Fig. S18) and for peak purity results (Figure S19).

**Accuracy**

Accuracy for cinnarizine at lower concentrations was determined at six levels in the range of 0.05 to 5.0 %w/w of test concentrations. At higher concentrations, accuracy for cinnarizine was calculated at six levels in the range of 25 to 150 %w/w of test concentrations. The accuracy for all five specified impurities and two degradation products was determined at seven levels in the range of 0.05 to 2.0 %w/w of test concentrations. The accuracy of two antioxidants was checked and confirmed by covering the specified range at five concentration levels, such as 50, 70, 100, 130, and 150% for methylparaben and propylparaben. The recoveries for the cinnarizine at the lower level and for all impurities and degradation products were found to be in the accepted range of 80.0-120.0%. However, for cinnarizine and two antioxidants, the assay was within 98.0-102.0%. The determined relative response factor was considered for the calculation of the accuracy of all impurities and degradation products. The accuracy results for methylparaben (Table S8), propylparaben (Table S9), all impurities and degradation products (Table S10), and cinnarizine (Table S11) are provided in the supplementary information.

**Precision**

Three sub parameters, such as system repeatability, analysis repeatability, and intermediate precision, were evaluated to assess and confirm the precision parameter. Throughout the method validation, system repeatability was evaluated and confirmed as a percent RSD of five replicate injections of the same 100 percent standard solution, with pre-defined acceptance
criteria for percent RSD of ≤ 0.73%. The analysis repeatability was estimated as a percentage of RSD at each concentration level injected and calculated as an individual recovery in accuracy parameter. The intermediate precision was calculated by analyzing two separate sets of spiked samples on different instruments using different columns on different days with separate solution preparations. Six separate sample solutions of four different forms of cinnarizine were spiked with 0.50% of all impurities, and the degradation products were analyzed. The percent relative standard deviation for the assay of cinnarizine was found to be within 2.0%, and that for all impurities and degradation products was within 5.0%. The system repeatability results with respect to peak areas observed in 25% and 100% standard solution (Table S12) are provided in the supplementary information.

**Limit of detection and quantification (LOD and LOQ)**

The values for the limit of detection and limit of quantification for cinnarizine for all five specified impurities and two degradation products (eight analytes) were estimated experimentally by analyzing the spiked standard solution in the dilution solvent as a blank matrix. The LOD values for all eight analytes were set to the 0.03% level (0.1125 µg/mL) based on a signal-to-noise ratio greater than 3.0. The LOQ values for all eight analytes were set to the 0.05% level (0.1875 µg/mL) based on a signal-to-noise ratio greater than 10 and proved by accuracy and precision parameters. The chromatograms of spiked LOQ solution and LOD solution is shown in the supplementary information (Fig. S20 and S21).

**Linearity**

The linearity curve of the cinnarizine was plotted and confirmed at 0.05 to 150 %w/w of test concentrations (0.1875 to 562.5 µg/mL). While the linearity curve of all five specified impurities and two degradation products were plotted and confirmed at 0.05 to 2.0 %w/w of test concentrations (0.1875 to 7.5 µg/mL). The linearity curve for methylparaben (5.0 to 15.0 µg/mL) and propylparaben (1.25 to 3.75 µg/mL) was plotted and confirmed at 50 to 150 %w/w. For all ten analytes, the correlation of the concentration versus the responses was found to be linear, and the coefficient of determination ($R^2$) was about ≥ 0.997. The percent relative standard deviation (%RSD) of the response factor of all ten analytes was found to be below 8.9%. The validation parameters like accuracy, linearity, and analysis repeatability were evaluated separately for drug substances, tablets, capsules, and oral suspension formulations by preparing solutions without and with respective placebos and injecting them with a triplicate preparation. The overlay chromatogram and linearity graphs of all analytes are presented in supplementary information (Fig. S22 and S23).

**Range, solution stability, filtration study, relative response factor, and system suitability**

The range for cinnarizine was validated from 0.05 to 150% (0.1875 to 562.5 µg/mL). For all five impurities and two degradation products, it was from 0.05 to 2.0% (0.1875 to 7.5 µg/mL), and for methylparaben (5.0 to 15.0 µg/mL) and for propylparaben (1.25 to 3.75 µg/mL) from 50 to 150%. The stability of solutions was proven on the solutions of system suitability tests, standards, and sample solutions and was found to be stable at room temperature for seven days. The filtration study was demonstrated on the unspiked and spiked sample solutions of the tablet, capsule, and oral suspension formulations. It concluded that there were no interfering contaminants extracted from the filter and that there was no significant adsorption of any analyte under investigation onto the filter bed. The relative response factors were calculated from the slopes of the linearity curves of all impurities and degradation products in the specified range and the slope of cinnarizine in the assay range. The outcome of range, solution stability, filtration study, and relative response factor are listed in Table 2. The system suitability parameters are generated for each sample set during the entire method validation and every time it meets the pre-defined acceptance criteria, as shown in supplementary information (Table S13).
### Table 2. Validation outcome of the UHPLC method (for quantification) and outcome of UHPLC-ESI-MS method (for confirmation)

| Parameter                        | API          | Impurity- A | Impurity- B | Impurity- C | Impurity- D | Impurity- E | Degradation Product | Anti-oxidants |
|----------------------------------|--------------|-------------|-------------|-------------|-------------|-------------|---------------------|--------------|
| Cinnarizine                      |              |             |             |             |             |             | Cinnamyl Piperazine | Benzhydrol    |
| Impurity- A                      | 3.7          | 7.2         | 7.8         | 11.9        | 14.7        | 2.2         | 5.2                 | 2.9          |
| Impurity- B                      | 6.5          | 3.7         | 7.2         | 7.8         | 11.9        | 14.7        | 2.2                 | 5.2          |
| Impurity- C                      | 1.00         | 1.11        | 1.20        | 1.83        | 2.26        | 0.34        | 0.80                | 0.45         |
| Impurity- D                      | 0.1125       | 0.1125      | 0.1125      | 0.1125      | 0.1125      | 0.1125      | 0.1125              | 0.1125       |
| Impurity- E                      | 0.1875       | 0.1875      | 0.1875      | 0.1875      | 0.1875      | 0.1875      | 0.1875              | 0.1875       |
| Benzhydrol                       | 562.5        | 7.50        | 7.50        | 7.50        | 7.50        | 7.50        | 7.50                | 7.50         |
| Methyl- paraben                  | 2.2          | 5.2         | 2.9         | 4.7         |             |             |                     |              |
| Propyl- paraben                  | 0.7          | 1.2         | 0.39        | NA          |             |             |                     |              |
| Regression equation              | y = 14234.3  | y = 10149.4 | y = 14325.6 | y = 17869.9 | y = 12760.9 | y = 11797.3 | y = 5563.8          | y = 6715.1    |
| Coefficient of determination (R^2) | 1.00         | 1.00        | 0.999       | 1.00        | 0.997       | 1.00        | 1.00                | 1.00         |
| % RSD for Response factor        | 3.0          | 5.8         | 2.9         | 7.3         | 7.6         | 5.4         | 3.6                 | 5.0          |
| Linearity range (µg/mL)          | 0.1875 - 562.5 | 0.1875 - 7.5 | 0.1875 - 7.5 | 0.1875 - 7.5 | 0.1875 - 7.5 | 0.1875 - 7.5 | 5.0 - 15.0          | 1.25 - 3.75  |
| Stability of solutions (at RT)   | 7 Days       | 7 Days      | 7 Days      | 7 Days      | 7 Days      | 7 Days      | 7 Days              | 7 Days       |
| Filter for Sample filtration a   | 0.45µm       | 0.45µm      | 0.45µm      | 0.45µm      | 0.45µm      | 0.45µm      | 0.45µm              | 0.45µm       |
| Relative Response Factor         | 1.00         | 0.72        | 1.26        | 1.00        | 1.12        | 0.90        | 0.83                | 0.39         |
| Purity Angle                     | 0.085        | 1.054       | 0.471       | 0.207       | 0.269       | 1.971       | 0.235               | 0.033        |
| Purity Threshold                 | 0.364        | 1.618       | 0.841       | 0.473       | 0.559       | 3.06        | 0.531               | 1.721        |
| Molecular formula                | C_26 H_28 N_2 | C_17 H_20 N_2 | C_26 H_28 N_2 | C_17 H_20 N_2 | C_17 H_20 N_2 | C_26 H_28 N_2 | C_17 H_20 N_2 | C_26 H_28 N_2 |
| Exact mass [M] (g/mol)           | 368.2        | 252.2       | 368.2       | 520.3       | 484.3       | 418.2       | 202.2               | 184.1        |
| Molecular ion [M ± H] +          | [M + H]^+    | [M + H]^+   | [M + H]^+   | [M + H]^+   | [M + H]^+   | [M + H]^+   | [M + H]^+           | [M + H]^+     |
| m/z value in Standard            | 369.36       | 253.26      | 369.36      | 485.44      | 485.46      | 419.42      | 203.26              | 185.09       |
| m/z value in Sample              | 369.37       | 253.28      | 369.36      | 485.43      | 485.44      | 419.43      | 203.27              | 185.09       |

NA: Not Applicable, a: Pall, Acrodisc CR 25mm syringe filter with 0.45µm PTFE membrane, part No. 4219T filter, RT: Room temperature
Applicability of proposed methods and comparison with reported methods

To demonstrate the applicability of proposed methods, we analyzed individual stock solutions of all ten analytes, all sample solutions (unsiked and spiked) of cinnarizine drug substance, cinnarizine 25 mg tablets, cinnarizine 75 mg capsules, and cinnarizine 75 mg/mL oral suspension by proposed methods. All the samples were analyzed using the UHPLC method for simultaneous quantification of all ten analytes and the UPLC-PDA-ESI-MS method for correct identification and confirmation of results. The PDA and MS data of all unspiked sample solutions revealed that the cinnamyl piperazine and benzhydrol were not detected, while the concentrations of impurity-A, impurity-B, impurity-C, impurity-D, and impurity-E were quite low. The assay of cinnarizine was calculated with a 25% sample solution and a 100% sample solution, and the results showed that there were no significant differences between these results. While the content of all impurities was calculated in the stock sample solution against a 25% and 100% standard solution, the results showed that there were no significant differences between these results. To confirm and compare the results observed by the proposed UHPLC method, all sample solutions were also analyzed as per the reported HPLC method. The observed results of the reported method and the proposed methods are presented in supplementary information (Table S14) and overlay chromatogram (Fig. S24). Based on these observed results, it was concluded that there were no significant differences in results generated by the reported method and the proposed methods. However, the generated chromatogram and results confirmed the higher sensitivity and detection efficiency with the significance of the proposed methods. The results obtained from mass spectral data were confirmed to have all the peaks observed in the sample chromatogram by the UHPLC-PDA data. All peaks were pure, and no coelution was observed. The consistency of observed results from mass spectra in all samples was demonstrated by comparing the values of molecular weights observed with the stock standard solution of all analytes. The consistency of observed m/z values with UPLC-ESI-MS of all ten analytes was also confirmed with expected m/z values with ESI-MS, extracted from ChemDraw Software. In mass spectral data, both antioxidants (methylparaben and propylparaben) were identified in negative mode, while all five specified impurities (Impurity-A to E), two degradation products (cinnamyl piperazine and benzhydrol) and API (cinnarizine) were identified in positive mode. The UV absorption spectra recorded by the PDA detector, mass spectra, data taken from ChemDraw Professional, and overlay chromatograms for all ten analytes are shown in supplementary information (Fig. S25 to S35).

Thus, compared to previously published and reported methods, the main benefits and selectivity of the proposed methods are: i) the quantitative and qualitative applicability of the methods in four different pharmaceutical forms (drug substances, tablets, capsules, and oral suspension); ii) the first UHPLC method for simultaneous quantification of all ten analytes at a single wavelength; iii) it offers very high sensitivity; iv) faster analysis; v) lower LOD and LOQ levels; vi) greater ability to reduce chemical and solvent waste, analysis time, and analysis cost; and vii) due to the specific nature of the method, it can be used for assay and purity analysis of all starting material, intermediates, specified impurities, and degradation products.

Conclusions

The proposed UHPLC method is a reverse-phase liquid chromatographic method compatible with mass spectrometers and has been concluded to be a rapid, timesaving, cost effective, linear, and robust method for accurate and precise quantification. With different approaches, it is very useful and suitable for the simultaneous quantification of cinnarizine, its five specified impurities, two degradation products, and two antioxidants by the UHPLC technique for the first time. The analysis of the samples with the UPLC-PDA-ESI-MS method additionally offers correct identification, confirmation of results, and is useful for the investigation of unknown peaks or contaminants. The proposed UHPLC
method was found to be more capable of reducing chemical and solvent waste, analysis time, and cost of analysis with improved sensitivity in chromatographic analysis. It is a single, useful, and common method for different formulations of cinnarizine, like cinnarizine drug substance and cinnarizine drug products (tablets, capsules, and oral suspension). In other words, the presented methods are more helpful for comprehensive quality evaluation of cinnarizine than separate methods to quantify assays of API, impurities, and antioxidants.

Declaration of competing interest
The authors declare that they have no competing financial interests or personal relationships that could have seemed to impact the work reported in this research paper.

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