Development and Validation of an UPLC Method for Rapid Determination of Ibuprofen and Diphenhydramine Citrate in the Presence of Impurities in Combined Dosage Form

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Abstract

A novel, stability-indicating gradient reverse-phase ultra-performance liquid chromatographic method was developed for the simultaneous determination of ibuprofen and diphenhydramine citrate in the presence of degradation products and process related impurities in combined dosage form. The method was developed using C18 column with mobile phase containing a gradient mixture of solvent A and B. The eluted compounds were monitored at 220 nm. Ibuprofen and diphenhydramine citrate were subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal, and photolytic degradation. Major unknown impurity formed under oxidative degradation was identified using LC–MS–MS study. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantitation, accuracy, precision and robustness. The described method was linear over the range of 0.20–6.00 µg/mL ($r > 0.998$) for Ibuprofen and 0.084–1.14 µg/mL for diphenhydramine citrate ($r > 0.998$). The limit of detection results were ranged from 0.200–0.320 µg/mL for ibuprofen impurities and 0.084–0.099 µg/mL for diphenhydramine citrate impurities. The limit of quantitation results were ranged from 0.440 to 0.880 µg/mL for ibuprofen impurities and 0.258 to 0.372 µg/mL for diphenhydramine citrate impurities. The recovery of ibuprofen impurities were ranged from 98.1% to 100.5% and the recovery of diphenhydramine citrate impurities were ranged from 97.5% to 102.1%. This method is also suitable for the simultaneous assay determination of ibuprofen and diphenhydramine citrate in pharmaceutical dosage forms.

Introduction

Ibuprofen {(2RS)-2-[4-(2-Methylpropyl)phenyl]propanoic acid} (I) is a nonsteroidal anti-inflammatory drug, which is available in 400 mg, 600 mg, and 800 mg tablets for oral administration. I is indicated for relief of the signs and symptoms of rheumatoid arthritis and osteoarthritis, for relief of mild to moderate pain and also indicated for the treatment of primary dysmenorrhea (1).

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Diphenhydramine citrate (2-(Diphenylmethoxy)-N, *N*-dimethylethylamine citrate) (DC) is an antihistamine drug and it blocks the effects of the naturally occurring chemical histamine in the body. It is used to treat sneezing, runny nose, itching, watery eyes, hives, rashes, itching, and other symptoms of allergies and the common cold. It is also used to suppress coughs, to treat motion sickness, to induce sleep, and to treat mild forms of Parkinson's disease (2). It is available in 25 mg and 50 mg caplets. It is also available in capsules as Diphenhydramine hydrochloride for oral administration. In combination these are available in 200/38 mg of I and DC, respectively. As far as could be determined, only one liquid chromatography (LC) method (3) was reported for simultaneous determination of I and DC from combined tablets, but it is out of scope because it deals with isocratic assay estimation of ibuprofen and diphenhydramine citrate. It also did not separate and determine the impurities and degradants formed from the force degradation study. It requires gradient elution for separation of closely related impurities of ibuprofen and diphenhydramine citrate.

Ultra-performance (UP) LC is a recent technique in liquid chromatography, which enables significant reductions in separation time and solvent consumption. Literature indicates that a UPLC system allows approximately nine fold decreases in analysis time as compared to the conventional high-performance (HP) LC system using 5 µm particle size analytical columns, and approximately threefold decrease in analysis time in comparison with 3 µm particle size analytical columns without compromise on overall separation (4–8).

The present research work was to develop a suitable single stability indicating UPLC method for simultaneous determination of I and DC in the presence of degradation products and process related impurities from combined dosage form. The developed LC method was validated with respect to specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy, and robustness. Forced degradation studies were performed on the tablets to show the stability indicating nature of the method and also to ensure the compliance in accordance with ICH guidelines. One major degradation product was observed from the oxidative stress study. This impurity was identified as diphenhydramine citrate impurity

based on LC–MS study and literature survey also reveals that it has not been reported elsewhere. Impurities IP-1, IP-2, IP-3, IP-4, IP-5, and IP-6 are process related impurities of ibuprofen and moreover IP-1, IP-2, IP-3, and IP-4 impurities are listed as impurity A, B, C, and D in European pharmacopeia (9) and DC-1 and DC-2 are diphenhydramine citrate impurities. The chemical structures of I and DC and their eight impurities are presented in Figure 1.

Experimental

Chemicals and reagents

The purity of all chemicals was above 97%. I and DC combined tablets (38 mg of DC and 200 mg of I) and standards of I (99.8%), DC (100.0%) and their eight impurities namely IP-1 (99.1), IP-2 (97.5), IP-3 (99.2), IP-4 (99.0), IP-5 (98.3), IP-6 (97.6), DC-1 (100.0), and DC-2 (98.2) were supplied by Dr. Reddy's Laboratories, Ltd. (Hyderabad, India). The HPLC grade acetonitrile and analytical grade KH_2PO_4 and ortho phosphoric acid and triethylamine were purchased from Merck (Darmstadt, Germany). High purity water was prepared by using Milli Q Plus water purification system (Millipore, Milford, MA).

Equipment

The Acquity UPLC system (Waters, Milford, MA) used consisted of a binary solvent manager, a sample manager, and a photo diode array (PDA) detector. The output signal was monitored and processed using empower2 software. A Cintex digital water bath (Mumbai, India) was used for hydrolysis studies. Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex). The pH of the solutions was measured by a pH meter (Thermo Orion Model 420 A, Worcester, MA). All solutions were degassed by ultra sonication (Power sonic 420, Labtech, Seoul, Korea) and filtered through a 0.45-µm Nylon 6,6 filter (PALL Life Sciences, Port Washington, NY).

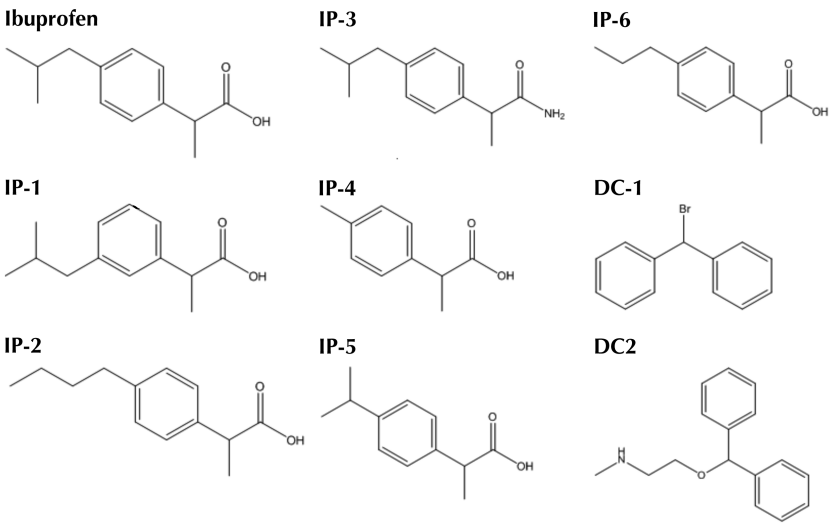

Figure 1. Structures of ibuprofen and diphenhydramine citrate, and their eight impurities.

Chromatographic conditions

The method was developed using Waters Aquity BEH C18, 50 × 2.1 mm; 1.7 µm column with mobile phase containing a gradient mixture of solvent A and B. 0.1% triethylamine buffer, pH adjusted to 3.2 with phosphoric acid was used as a mobile phase buffer. Buffer and acetonitrile in the ratio 80:20, v/v; was used as solvent A and buffer and acetonitrile in the ratio 50:50, v/v; was used as solvent B. The gradient program (T/%B) was set as 0/0, 7.5/50, 17/50, 17.5/0, and 20/0. The mobile phase was filtered through a nylon 0.45-µm membrane filter. The flow rate of the mobile phase was 0.4 mL/min. The column temperature was maintained at 25°C and the wavelength was monitored at 220 nm. The injection volume was 2 µL.

LC–MS–MS conditions

LC–MS–MS system (Waters 2695 Alliance LC coupled with quattromicro MS with Mass Lynx software, Waters) was used for the unknown compounds formed during forced degradation studies. Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm and 5µm particle size (Agilent Technologies, USA) was used as stationary phase. Solvent A is formic acid buffer (pH adjusted to 3.2 with formic acid) and acetonitrile in the ratio 80:20, v/v and solvent B is formic acid buffer (pH adjusted to 3.2 with formic acid) and acetonitrile in the ratio 50:50, v/v, with gradient programme: time (t)/% solvent B: 0/0, 30/50, 45/50, 80/50, 82/0, 90/0. Solvent B was used as diluent. The flow rate was 1.0 mL/min. The analysis was performed in positive electro spray positive ionization mode. Capillary and cone voltages were 3.5 kV and 25 V, respectively. Source and dissolvation temperatures were 120 and 350°C, respectively. Dissolvation gas flow was 650 L/h.

Preparation of stock solutions

A stock solution of I and DC (4000 µg/mL of I and 760 µg/mL of DC) was prepared by dissolving appropriate amount of drugs in solvent B. Working solutions of 2000 µg/mL of I and 380 µg/mL of DC, and 500 µg/mL of I, and 95 µg/mL of DC were prepared from the previously described stock solution for related substance determination and assay determination, respectively. A stock solution of impurity (mixture of IP-1, IP-2, IP-3, IP-4, IP-5, IP-6, DC-1 and DC-2) at 0.5 mg/mL was prepared in solvent B.

Preparation of sample solution

Tablet powder (200/38 mg tablets) equivalent to 38 mg of DC (200 mg of I) drug was dissolved in Solvent B with sonication for 20 min to give a solution containing 2000 µg/mL of I and 380 µg/mL of DC, and 2.5 mL of this solution was diluted to 10 mL with solvent B, to give a solution containing 500 µg/mL of I and 95 µg/mL of DC. These solutions were filtered through a 0.45-µm pore size Nylon 66 membrane filter.

Method validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The methods were validated according to International Conference

on Harmonization Q2 (R1) guidelines (10) for validation of analytical procedures in order to determine the specificity, linearity, LOD, LOQ, accuracy, precision, and robustness.

Solution stability

The stability of I and DC in solution was determined by leaving test solutions of the sample and reference standards in tightly capped volumetric flasks at room temperature for 48 h during which they were assayed at 12 h intervals. The % assay of the results was calculated for solution-stability experiment. The stability of I and DC and their impurities in solution for related substance method was determined by leaving spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h and measuring the amounts of the five impurities at every 12 h.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (11). The specificity of the developed LC method for I and DC was carried out in presence of its eight impurities. Stress studies were performed at an initial concentration 2000 µg/mL of I and 380 µg/mL of DC on tablets to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress condition of UV light (254 nm), heat (105°C for 12 h), acid (2N HCl at 25°C for 2 h), base (5N NaOH at 50°C for 2 h), hydrolytic (50°C for 12 h), and oxidation (3.0% H2O2 at 40°C for 2 h) to evaluate the ability of the proposed method to separate I and DC from their degradation products. Peak purity tests were carried out for I and DC peaks by using PDA detector for stress samples.

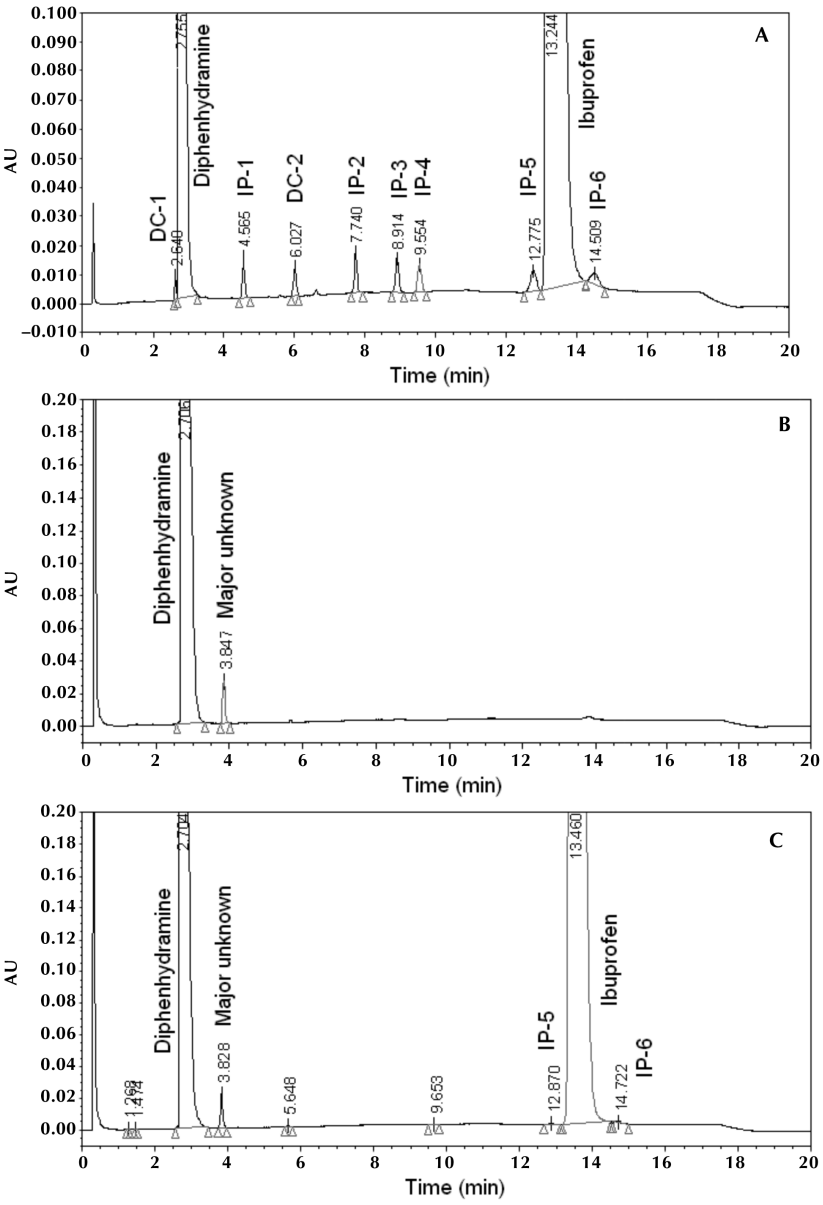

Figure 2. Typical chromatograms of (A) ibuprofen and diphenhydramine citrate spiked with their impurities, (B) oxidative stress of diphenhydramine citrate, and (C) oxidative stress of ibuprofen and diphenhydramine citrate tablets.

Linearity

Linearity test solutions for the assay method were prepared from I and DC stock solutions at five concentration levels from 50% to 150% of assay concentration (250, 375, 500, 625, and 750 µg/mL for I and 47, 71, 95, 118, and 143 µg/mL for DC). The peak area versus concentration data was treated by least-squares linear regression analysis. Linearity test solutions for I and DC and their impurities were prepared by diluting stock solutions to required concentrations. The solutions were prepared at six concentration levels from LOQ to 150% of the specification level 0.2% (LOQ, 0.075, 0.15, 0.20, 0.25, and 0.30%).

LOD and LOQ

The LOD and LOQ for I and DC and their impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations (10). Precision study was also carried out at the LOQ level by injecting six individual preparations and calculated %RSD of the area.

Accuracy

The accuracy of the assay method was evaluated in triplicate using three concentration levels 50, 100, and 150 µg/mL on tablets (200/38 mg tablets). Standard addition and recovery experiments were conducted on real sample to determine accuracy of the related substance method. The study was carried out in triplicate using four concentration levels namely LOQ, 0.10%, 0.20%, and 0.30%. The percentages of recoveries for I and DC and their impurities were calculated.

Precision

The precision of the method was verified by repeatability and by intermediate precision.

Table I. Chromatographic Performance Data

Compound	RT (Min)	RRT *	Resolution	Tailing factor
DC-1	2.64	0.96	–	1.0
DPHD†	2.75	1.00	2.37	1.2
IP-1	4.56	0.34	8.87	1.1
DC-2	6.02	2.18	10.92	1.1
IP-2	7.74	0.58	11.86	1.0
IP-3	8.91	0.67	7.15	1.1
IP-4	9.55	0.72	3.22	1.1
IP-5	12.77	0.96	11.99	1.0
Ibuprofen	13.24	1.00	1.08	1.2
IP-6	14.50	1.09	2.65	1.1

* Relative retention times (RRT) for DC-1 and DC-2 were calculated against the retention time (RT) of Diphenhydramine and RRT for IP-1, IP-2, IP-3, IP-4, IP-5 and IP-6 were calculated against the retention time (RT) of ibuprofen.
† Diphenhydramine.

Table II. Summary of Forced Degradation Studies

Stress condition (degradation)	% impurities formed								% degradation
	DC-1	DC-2	IP-1	IP-2	IP-3	IP-4	IP-5	IP-6	
Oxidative	ND*	ND	ND	ND	ND	ND	0.07	0.06	5.13
Acid	0.11	ND	ND	ND	ND	ND	0.04	ND	0.35
Base	0.09	ND	ND	ND	ND	ND	0.05	ND	0.32
Hydrolytic	0.10	ND	ND	ND	ND	ND	0.06	ND	0.22
Thermal	3.2	ND	ND	ND	ND	ND	0.05	ND	3.5
Photolytic	0.08	ND	ND	ND	ND	ND	ND	ND	0.18

*ND: Not Detected.

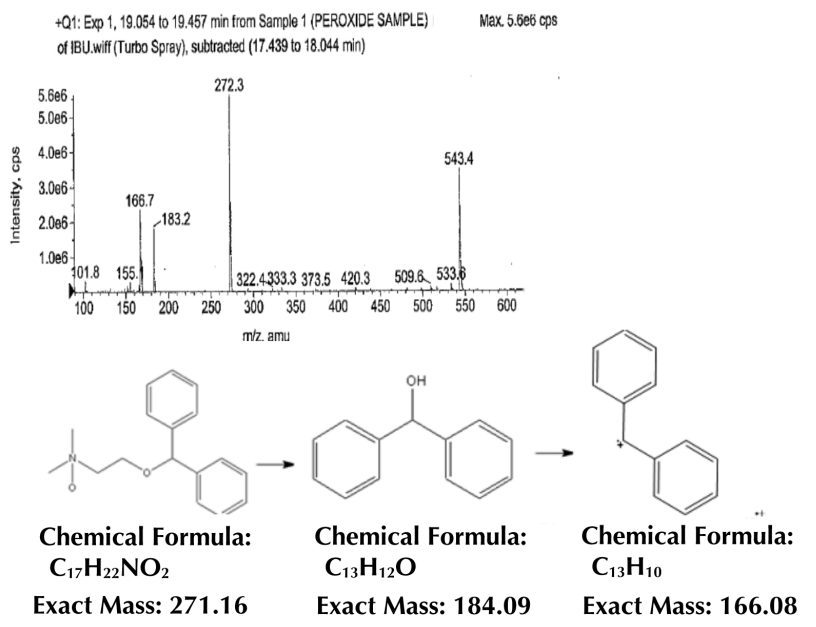

Figure 3. Mass spectrum of 3.8 min RT degradation product formed in oxidative degradation of diphenhydramine citrate.

Repeatability was checked by injecting six individual preparations of I and DC on real sample (200/38 mg tablets) spiked with 0.20% of its eight impurities. For this test, 0.2% of I impurities were spiked with respect to I concentration 2000 µg/mL and 0.2% of DC impurities were spiked with respect to DC concentration 380 µg/mL. The %RSD of area for each impurity was calculated. The intermediate precision of the method was also evaluated using a different analyst and on a different day.

Assay method precision was evaluated by carrying out six independent assays of real sample of I and DC at 500 µg/mL of I and 95 µg/mL of DC against qualified reference standard. The intermediate precision of the assay method was evaluated by different analysts.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between I and DC impurities and tailing factor for I and DC and their impurities was recorded. The flow rate of the mobile phase was 0.4 mL/min. To study the effect of flow rate on the resolution, flow was changed by 0.1 units from 0.3 to 0.5 mL/min. The effect of the column temperature on resolution was studied at 20 and 30°C instead of 25°C. The effect of the percent organic strength on resolution was studied by varying acetonitrile by –5% to +5%. The effect of pH of mobile phase buffer was studied on resolution by varying pH ± 0.1 units of method pH (3.2) while other mobile phase components were held constant as stated previously.

Results and Discussion

Method development and optimization

The main target of the chromatographic method was to get the separation of impurities, namely IP-1, IP-2, IP-3, IP-4, IP-5, IP-6, DC-1, DC-2, and the degradation products generated during stress studies, from the analyte peaks. Impurities were co-eluted by using different stationary phase like C8 (Waters Aquity BEH C8, 50 × 2.1 mm; 1.7 µm column) and phenyl (Waters Aquity BEH phenyl, 50 × 2.1 mm; 1.7 µm column) and different mobile phases containing buffers like phosphate, sulphate and acetate with different pH (7.0) and using organic modifiers like acetonitrile, methanol, and ethanol in the mobile phase. Apart from the co-elution of impurities poor peak shapes for some impurities were also noticed. Ortho phosphoric acid buffer with pH 3.2 and acetonitrile (solvent A was buffer, acetonitrile in

the ratio 80:20 v/v and solvent B was buffer, acetonitrile in the ratio 30:70 v/v) at 0.4 mL/min flow was chosen for initial trail with a Waters Aquity BEH C18, 50 × 2.1 mm; 1.7 µm column. When the impurity spiked sample was injected, the resolution between impurities and analytes was poor and also poor peak shapes for both actives drugs I and DC were noticed.

To get the good resolution of impurities from analyte, triethylamine (0.1%) was added to water and then pH adjusted to 3.2 with ortho phosphoric acid. The resolution was good among impurities and analytes. The effect of buffer pH was also studied under the previously described conditions and it was found that at higher and lower pH, the tailing of the active drugs I and DC peaks was more and also resolution was poor between compounds. The percentage of acetonitrile in the solvent B was also studied, when 70% and 60% of acetonitrile was used in solvent B, the resolution of IP-5and IP-6 from drug I was poor.

The results clearly indicated that on Waters Aquity BEH C18, 50 × 2.1 mm; 1.7 µm column and using solvent A (0.1% triethylamine buffer pH adjusted to 3.2 with ortho phosphoric acid and acetonitrile in the ratio 80:20, v/v) and solvent B (0.1% triethylamine buffer pH adjusted to 3.2 with ortho phosphoric acid and acetonitrile in the ratio 50:50, v/v), with gradient programme: time (*t*)/% solvent B: 0/0, 7.5/50, 17/50, 17.5/0, and 20/0 at detection wavelength 220 nm was successful in separation of both drugs from its impurities and degradation products and also eluted I, DC and their impurities as symmetrical peaks (Figure 2 and Table I). Interference from the excipients was also checked, no interference was observed.

Table III. Regression and Precision Data

Parameter	DC-1	DC-2	IP-1	IP-2	IP-3	IP-4	IP-5	IP-6
LOD (µg/mL)	0.099	0.084	0.200	0.200	0.280	0.320	0.280	0.280
LOQ (µg/mL)	0.372	0.258	0.560	0.440	0.760	0.880	0.520	0.520
Regression equation (*y*)								
Slope (b)	20021.2	33339.1	23878.8	25440.8	21322.2	22827.8	22991.9	23070.3
Intercept (a)	–631.8	–1903.3	–5468.3	–2723.9	–2143.3	–2747.7	–7722.4	–7507.8
Correlation coefficient	0.9994	0.9984	0.9987	0.9984	0.9981	0.9994	0.9995	0.9991
Precision*	0.45	0.57	1.60	2.01	0.60	1.05	2.2	2.4
Intermediate precision*	0.48	0.59	0.85	1.82	1.15	1.32	1.38	1.25

*(% RSD) #

Validation of the method

Solution stability

Assay (%) of both drugs during solution stability experiments was within ± 1%. The variability in the estimation of I and DC impurities was within ± 10% during solution stability experiment. The results from solution stability experiments confirmed that standard and sample solutions were stable up to 48 h for related substances analysis.

Results of forced degradation studies

All forced degradation samples were analyzed at an initial concentration 2000 µg/mL of I and 380 µg/mL of DC with LC conditions mentioned in the "Chromatographic conditions" section using PDA detector to ensure the homogeneity and purity of I and DC peaks. Significant degradation of I and DC was observed in oxidative (3.0% H_2O_2 at 40°C for 2 h) condition leading to the formation of major unknown at RT 3.8 min (Figure 2B and 2C). Oxidative stress was performed for DC alone and confirmed that major unknown at 3.8 min is degradant of DC. Mild degradation was observed in thermal (105°C for 12 h) condition leading to the formation DC-1 impurity. I and DC was found to be stable under hydrolytic (50°C for 12 h), acid (2N HCl at 25°C for 2 h), base (5N NaOH at 50°C for 2 h) and photolytic (10 days) degradation conditions.

The peak purity test results derived from photo diode array detector (PDA) confirmed that I and DC peaks were pure and homogeneous in all the analyzed stress and thus confirms the stability-indicating power of the developed method. Results of forced degradation studies are reported in Table II.

Identification of major degradation product (at 3.8 min RT) formed in oxidative stress

LC–MS–MS analysis was carried out for the oxidative stress sample of I and DC using Waters 2695 Alliance mass spectrometer with conditions mentioned in the "LC–MS–MS conditions" section. The degradation product formed at 3.8 min RT shows the mass of 272 which was 16 higher mass than DC mass 256. The fragmentation pattern (Figure 3) clearly indicated that formed degradant was *N*-oxide of DC which was supported by chemical properties of

Table IV. Evaluation of Accuracy

Amount spiked*	% Recovery†									
	I	DC	DC-1	DC-2	IP-1	IP-2	IP-3	IP-4	IP-5	IP-6
LOQ	98.5 ± 0.8	98.7 ± 0.3	98.1 ± 0.3	98.0 ± 0.8	98.1 ± 0.3	99.6 ± 0.3	99.1 ± 0.3	99.1 ± 0.3	99.7 ± 0.1	99.3 ± 1.2
50%	100.1 ± 0.2	99.8 ± 0.2	99.5 ± 0.9	98.5 ± 0.5	98.8 ± 0.1	100.2 ± 0.8	100.2 ± 1.1	100.1 ± 0.6	99.9 ± 0.3	99.9 ± 0.3
100%	99.1 ± 0.3	98.8 ± 0.4	97.5 ± 0.4	99.8 ± 0.3	100.5 ± 0.1	99.6 ± 0.5	99.0 ± 0.2	99.1 ± 0.2	98.4 ± 0.3	98.1 ± 0.5
150%	100.5 ± 0.5	99.1 ± 0.2	99.0 ± 0.2	102.1 ± 0.3	98.8 ± 0.4	98.1 ± 0.2	98.1 ± 0.2	98.3 ± 0.1	100.5 ± 0.8	99.5 ± 0.5

* Amount of five impurities spiked with respect to 0.20 % specification level individually to ibuprofen (I) and diphenhydramine citrate (DC).
† Mean ± %RSD for three determinations.

DC. The fragment 183.2 formed from the cleavage of –O–C bond and the fragment 166.7 results from the cleavage of –C–O-H. So the probable structure is as shown in Figure 3.

Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested and correlation coefficient obtained was greater than 0.999 for both I and DC. Linear calibration plot for impurities was obtained over the calibration ranges tested (i.e., LOQ to 0.30% for impurities). The correlation coefficient obtained was greater than 0.998 (Table III). The previously described results show that an excellent correlation existed between the peak area and the concentration of all eight impurities.

LOD and LOQ

The determined LOD, LOQ, and precision at LOQ values for I and DC and its five impurities are reported in Table III.

Accuracy

The percentage recovery of I and DC from tablets ranged from 98.5% to 100.5% for I and from 98.7 to 99.8% for DC. The percentage recovery of impurities in I and DC samples varied from 97.5% to 102.1%. The % recovery values for I and DC and their impurities are presented in Table IV. The LC chromatogram of spiked sample at 0.20% level of all eight impurities in I and DC tablets sample is shown in Figure 2.

Precision

The %RSD of assay of I and DC during the assay method repeatability study was 0.81, and 0.25% for I and DC; respectively. The %RSD for the area of IP-1, IP-2, IP-3, IP-4, IP-5, IP-6, DC-1 and DC-2 in related substance method repeatability study was within 2.01%. The %RSD of the assay results obtained in the intermediate precision study was within 0.75% for both I and DC. The %RSD for the area of IP-1, IP-2, IP-3, IP-4, IP-5, IP-6, DC-1 and DC-2 were well within 1.32% for intermediate precision, conforming good precision of the method. The %RSD values are presented in Table III.

Robustness

In all the deliberate varied chromatographic conditions like flow rate (± 0.1 mL/min of 0.4 mL/min), column temperature (± 5°C of 25°C), composition of organic solvent (± 5% of method organic solvent) and pH of mobile phase buffer (0.1 unit of pH 3.2), all analytes were adequately resolved and elution orders remained unchanged. The resolution between all pair compounds was greater than 2.0 and tailing factor for I and DC and their impurities was less than 1.2. The variability in the estimation of I and DC impurities was within ± 10%.

Conclusions

The rapid reproducible gradient RP-UPLC method developed for quantitative analysis of I and DC and related substances in pharmaceutical dosage forms is precise, accurate, linear, robust, and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of I and DC in combined dosage form.

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