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ORIGINAL ARTICLE



Stability-indicating assay method for determination of actarit, its process related impurities and degradation products: Insight into stability profile and degradation pathways[☆]

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Abstract The stability of the drug actarit was studied under different stress conditions like hydrolysis (acid, alkaline and neutral), oxidation, photolysis and thermal degradation as recommended by International Conference on Harmonization (ICH) guidelines. Drug was found to be unstable in acidic, basic and photolytic conditions and produced a common degradation product while oxidative stress condition produced three additional degradation products. Drug was impassive to neutral hydrolysis, dry thermal and accelerated stability conditions. Degradation products were identified, isolated and characterized by different spectroscopic analyses. Drug and the degradation products were synthesized by a new route using green chemistry. The chromatographic separation of the drug and its impurities was achieved in a phenomenex luna C18 column employing a step gradient elution by high performance liquid chromatography coupled to photodiode array and mass spectrometry detectors (HPLC–PDA–MS). A specific and sensitive stability-indicating assay method for the simultaneous determination of the drug actarit, its process related impurities and degradation products was developed and validated.

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1. Introduction

Actarit is an orally active immunomodulator used in the treatment of rheumatoid arthritis. It suppresses secondary inflammation by activation of Lyt-2+ cells and shows prophylactic and therapeutic effects on secondary inflammation in adjuvant arthritis in rats [1,2]. It prevents the progression of articular lesions [3] and also curtails type II and type IV allergic reactions in mice [4–6].

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Several high performance liquid chromatography (HPLC) methods have been reported in the literature for analysis of the drug actarit; however, they are mainly related to its determination in biological fluids [7–9]. Estimation of actarit in pharmaceutical formulation by HPLC was also reported [10] and the degradation of actarit in alkaline and acidic solutions was evaluated. The overall chemical stability of actarit was not sufficiently addressed and this method cannot be considered as a method for indicating stability. Photobiological properties of in vitro phototoxicological assays and photodegradation products of actarit were studied [11]. The chemical stability of actarit with respect to other stress conditions (oxidation, thermal) is presently unknown. An exhaustive study on the stability of actarit is demanding as the current International Conference on Harmonisation (ICH) guidelines require that stability analysis should be done by using stability-indicating assay methods (SIAM). After stress testing on the drug under various conditions, including hydrolysis (at various pH), oxidation, photolysis and thermal degradation and accelerated stress conditions, SIAM should be developed and validated [12].

Forced degradation studies were carried out on the drug in order to generate the potential degradation products under different stress conditions as per ICH guidelines. Two potential degradation products that were formed were isolated, characterized and synthesized. The structural characterization of the degradation product was determined using Fourier transform infrared spectroscopy (FTIR), liquid chromatography–tandem mass spectrometry (LC–MS/MS), proton-nuclear magnetic resonance (^1H NMR), carbon-nuclear magnetic resonance (^{13}C NMR), and hydrogen–deuterium exchange nuclear magnetic resonance (D_2O exchange NMR). Degradation products which were formed in smaller quantities in oxidative degradation were identified by high performance liquid chromatography coupled to photodiode array and mass spectrometry detectors (HPLC–PDA–MS).

Structural characterization of the major degradation products enables one to establish the degradation pathway under which the degradation products are formed. Synthesis of the drug assists in identifying the process related impurities. This further helps with the quantitative determination of the drug, in the presence of its process related impurities and degradation products [13].

In the present work, intrinsic stability of the drug actarit was found and a selective, precise and accurate HPLC–PDA–MS

method was developed for simultaneous determination of actarit, its process related impurities and degradation products. Rate of degradation of the drug was determined by performing hydrolysis of the drug at different temperature conditions.

2. Experimental

2.1. Reagents and chemicals

The drug actarit, its process related impurities (IMP) and degradation products (DP) were synthesized in the lab. Buffer salts and glacial acetic acid were purchased from Merck, India. Highly purified water for HPLC was obtained from Milli Q plus water purifying system, Millipore. Methanol and acetonitrile of HPLC grade were obtained from Fischer Scientific, India. Mobile phase was vacuum filtered through a $0.22\ \mu\text{m}$ poly-tetrafluoroethylene (PTFE) filter membrane and degassed using a sonicator to remove the dissolved gases. Chemical structures for the drug, process related impurities and degradation products are schematically represented in Fig. 1. Chemical names for the drug, impurities and degradation products are as follows: (A) Drug: p-acetylamino phenyl acetic acid, (B) IMP-1/DP-I: p-amino phenyl acetic acid, (C) IMP-2: p-amino acetophenone, (D) IMP-3: p-Acetylamino acetophenone, (E) IMP-4/DP-II: p-acetylamino toluene, (F) DP-III: p-amino toluene, (G) DP-IV: 2-phenyl-1,2-oxaziridine, (H) IMP-5: 2-(4-aminophenyl)-1-morpholino ethane thione, and (I) IMP-6: N-4-(2-morpholino-2-thioxoethyl)phenyl acetamide.

2.2. Instrumentation and chromatographic conditions

2.2.1. High pressure liquid chromatography (HPLC)

System configuration of Shimadzu HPLC system consisted of two LC-6AD pumps equipped with an SPD20 (PDA) detector, and was used throughout the analysis. Data analysis and interpretation were carried out using Class VP software (6.14 SP1). The analyses were carried out on a phenomenex luna C18 column ($250\ \text{mm} \times 4.6\ \text{mm} \times 10\ \mu\text{m}$). The measurements were carried out at a wavelength of 244 nm for the analytes.

The mobile phase was prepared in two solvent reservoirs A and B: Solvent reservoir A was a combination of aqueous ammonium

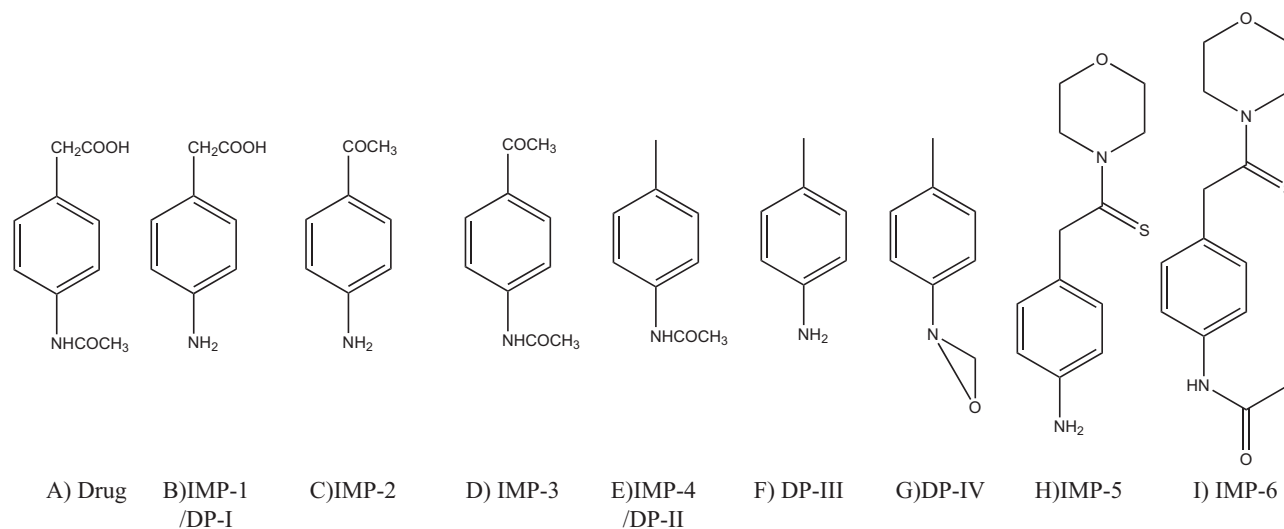


Fig. 1 Chemical structures for the drug actarit, its process related impurities and degradation products.

acetate buffer (pH 3.5; 10 mM, 450 mL), acetonitrile (25 mL) and methanol (25 mL) and solvent reservoir B was a mixture of methanol, acetonitrile and ammonium acetate buffer (pH 3.5, 10 mM) taken in an equal ratio. Flow rate used was 0.8 mL/min. The HPLC step gradient method used the following run time program: time (min)/solvent B concentration (%): 0.01–8.00/10, 8.01–15/20, 15.01–20.00/40, 20.01–26.00/60, 26.01–38.00/80, 38.01–40.00/60, 40.01–42.00/40, 42.01–43.00/20, and 43.01–45.00/10.

Semipreparative HPLC system has the same configuration as that of analytical except for the column, flow cell and fixed loop used. Semipreparative study [14,15] was performed with semipreparative phenomenex luna C18 column (250 mm × 10 mm × 10 µm), 0.5 mm path length flow cell and fixed loop 100 µL. Mobile phase used was the same. The flow rate used was 4.0 mL/min.

To find the degradation products which were formed in smaller quantities, the instrument used was HPLC–PDA–MS. The model used was Thermo LCQ fleet ion trap LC/MSⁿ. Nitrogen was used as the curtain gas and nebulizer gas. Collision induced dissociation was achieved by helium as the collision gas. The mass spectra were recorded in atmospheric pressure chemical ionization using the positive mode of detection. Mobile phase used was the same as that used in analytical chromatography. Stress samples of acid, alkaline, photolytic and oxidative degradation mixtures were subjected to HPLC–PDA–MS study to find out the degradation products which were formed in small quantities.

2.2.2. Accelerated conditions cabinet (ACC)

Humidity chamber (EIE Instruments Pvt. Ltd.) was employed to carry out accelerated stability testing at ACC conditions. The temperature was set at 40 ± 2 °C and the relative humidity at $75 \pm 5\%$.

2.3. Chromatographic behavior of impurities and degradation products

The behavior of the drug, impurities and degradation products under different pH conditions was studied. Ammonium acetate buffer (10 mM) was prepared in different pH values, ranging from 3.5 to 7 and the retentions of the impurities and degradation products were studied.

2.4. Forced degradation conditions

The drug was accurately weighed to 10 mg, then dissolved in a minimum amount of acetonitrile and made up to 10 mL by taking

either acid or base or water or 3% peroxide as per the requirement of the stress study [16] so as to make the concentration of the drug 1 mg/mL. Forced degradation conditions employed are tabulated in Table 1. In case of photolytic degradation, the drug was subjected to UV and visible light radiations in a photolytic chamber [17] (as per ICH guidelines). The drug sample was exposed to light for an overall illumination of 1.2 million lx h and an integrated near ultraviolet energy of 200 Wh/m². Thermal degradation studies were done by spreading a thin layer of the drug in a petri dish and keeping the temperature maintained at 70 °C for a period of 14 days. Before HPLC analysis, acidic samples were neutralized with 0.05 M base and then made up to 1 mL with the mobile phase; basic samples were neutralized by taking 0.1 mL of the reaction mixture with 0.05 M HCl and then made up to 1 mL with the mobile phase. In case of oxidative studies, the reaction mixture was lyophilized and then 10 mg of the compound was made up to 10 mL with acetonitrile. This solution was diluted 10 times with the mobile phase and then injected. Lyophilization procedure was done to prevent the injection of peroxide into the column. The addition of the mobile phase to the reaction mixture was to improve the peak shape in HPLC. The chromatograms of the drug under all studied stress conditions are represented in Fig. 2.

2.5. Rate of degradation kinetics

The drug was subjected to forced degradation studies using 0.1 M HCl at three different temperature conditions (60 °C, 70 °C and 80 °C) for a time period of 125 h. Similar experiments were carried out by changing the stress conditions to 0.1 M NaOH. Two different concentrations (1000 µg/mL and 100 µg/mL) were used to study the effect of concentration on the rate of degradation kinetics. This was done systematically by taking 100 µL of the solution from the reaction, neutralized and then made up to 1 mL with the mobile phase. An aliquot of 20 µL was injected and then analyzed. These experiments were conducted to investigate under which conditions (acidic or basic) the degradation was greater and also to find out whether the degradation is dependent on the concentration of the drug.

2.6. Stability of the drug

Stability of the drug was tested under different hydrolysis conditions starting from pH 1 to 14. Drug solution of concentration 1 mg/mL was prepared in buffer solutions starting from pH 1 to 14 individually and the solution was continuously stirred. The study was conducted for a period of 14 days at room temperature.

Table 1 Forced degradation condition applied for the drug.

Stress condition	DP-I	DP-II	% Assay	Mass balance	Remarks
Base hydrolysis (0.1M NaOH, 24h, 70 °C)	14.08	–	85.62	99.7	DP-I ^a
Acid hydrolysis (0.1M HCl, 24h, 70 °C)	9.70	–	90.10	99.8	DP-I ^a
Neutral hydrolysis (water, 14 days, 70 °C)	–	–	99.99	99.9	–
Oxidative degradation (3% peroxide) (continuous stirring, RT, 14 days)	–	13.8	85.00	98.8	DP-II ^a
Photolytic degradation (photolytic chamber, 21 days, 25 °C)	1.2	–	98.5	99.7	DP-I ^a
Thermal degradation (dry heat, 14 days, 70 °C)	–	–	99.98	99.98	–
Accelerated stability studies	–	–	99.7	99.7	–

Mass balance = % Assay of drug + % IMP + % DP by peak area.

^aMajor degradation product (DP) formed in the stress condition.

2.7. Synthesis of the drug and degradation products

2.7.1. Synthesis of DP-I (solvent free synthesis using Willgerodt–Kindler reaction) and the drug

p-Amino acetophenone (IMP-2) was acetylated with acetyl chloride followed by the addition of a saturated solution of sodium bicarbonate at lower temperature conditions. Once the reaction was completed, the reaction mixture was extracted with ethyl acetate. Ethyl acetate layer was separated, evaporated and the product formed (IMP-3) was isolated and subjected to Willgerodt–Kindler reaction. Willgerodt–Kindler reaction was carried out for 4 h at 120 °C in solvent free conditions [18]. The product thiomorpholide (IMP-5) (200 mg) was taken and refluxed with 10% alcoholic sodium hydroxide solution (25 mL). The progress of the reaction was monitored through thin layer chromatography

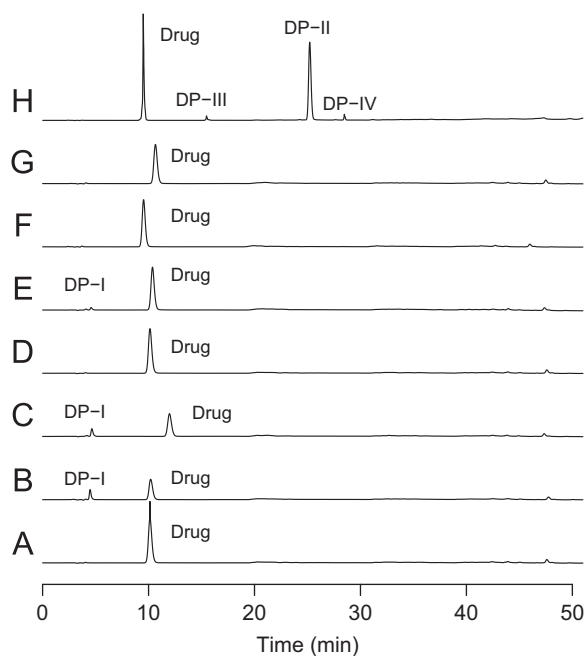


Fig. 2 HPLC chromatograms of the drug under different forced conditions: (A) drug; (B) acid stress conditions; (C) base stress conditions; (D) neutral stress conditions; (E) photolytic stress conditions; (F) thermal stress conditions; (G) accelerated stress conditions; and (H) oxidative stress conditions.

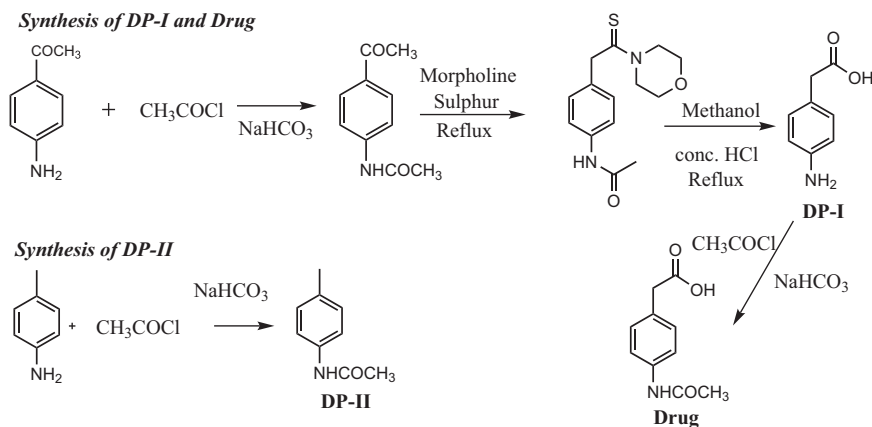


Fig. 3 Schematic representation of the synthesis of the degradation product DP-I, the drug and degradation product DP-II.

(TLC). Once the reaction was completed, the whole mixture was neutralized with acid (30% HCl) and lyophilized. The final lyophilized product p-amino phenyl acetic acid [DP-I] was recrystallized with methanol and water. In the above synthesis, it is necessary to acetylate p-amino acetophenone to get the desired product, thereby preventing the formation of N-oxide product (Fig. 3).

The product p-amino phenyl acetic acid (300 mg, 20 mM) (DP-I) was treated with acetyl chloride (350 mg, 24 mM) followed by the addition of a saturated solution of sodium bicarbonate at lower temperature conditions. Once the reaction was completed, the reaction mixture was extracted with ethyl acetate. Ethyl acetate layer was separated and evaporated to obtain the drug. The drug actarit was recrystallized with methanol. The melting point of the drug was found to be at 174 °C. The yield was found to be 70%. The product was characterized using mass and NMR spectroscopy. Instead of acetyl chloride, acetic anhydride can also be used. In case of acetic anhydride, yield of the drug was greater than 80% [19].

2.7.2. Synthesis of DP-II

p-Toluidine (500 mg, 4.6 mM) was acetylated using acetyl chloride (540 mg, 6.9 mM) in the presence of saturated sodium bicarbonate solution at lower temperature conditions to get the product under solvent free conditions (Fig. 3).

2.8. Sample preparation for HPLC method validation

2.8.1. Preparation of standard solutions

Solution A: 10 mg of actarit reference standard was weighed accurately and dissolved in a 10 mL volumetric flask with methanol. Solution B: A combined standard solution of accurately weighed degradation products DP I/Imp-I (10 mg), Imp-2 (10 mg), Imp-3 (10 mg), Imp-4 (10 mg)/DP II (10 mg), Imp-5 (10 mg), and Imp-6 (10 mg) was prepared in a 10 mL volumetric flask using methanol.

2.8.2. Solution stability

Solution stability of actarit in the related substance method was carried out by leaving analyte mixture solution (drug, degradation products and impurities) in a tightly capped volumetric flask at room temperature for 2 days. Content uniformity of impurities (IMP-1/DP-1, IMP-2, IMP-3, IMP-4/DP-2, IMP-5, and IMP-6) was checked in the test solutions [20].

3. Results and discussion

3.1. Optimization of mobile phase conditions

Method development of the drug in the preliminary studies involved the use of methanol along with the buffer. During forced degradation studies, chromatogram of the stressed mixtures showed co-eluting peaks. The same problem was encountered when acetonitrile was used alone with the buffer. The addition of acetonitrile along with methanol led to the resolution of the degradation products and the drug.

Analytes (process related impurities, degradation products, and the drug) were found to be a mixture of acidic, basic and neutral components. Hence, a modification in pH altered the separation selectivity for ionized or unionized solutes. Several trials were made with the mobile phase by varying the pH of the buffer and also by changing the proportions of the organic modifier [21].

Ammonium acetate buffer:acetonitrile:methanol (90:5:5, v/v/v, pump A) was used in combination with ammonium acetate buffer:acetonitrile:methanol (50:50:50 in pump B). The method had a total run time of 45 min using the step gradient method. In this combination, buffer pH was checked with 3.5 and further trials were done with pH values of 5–7. Better resolution was obtained by adjusting the pH of the buffer to 5, which illustrates the sensitivity of the polar compounds and the non-polar compounds in a less acidic environment (Fig. 4). Peak shape and system suitability parameters were improved as both the organic modifiers

were used in the mobile phase. Peak purity for each peak was found to be greater than 0.99. Mass spectra of the individual peaks (the drug, process related impurities and degradation products) which were obtained through HPLC–PDA–MS are shown in Fig. 5.

3.2. Mechanism

The drug follows an ionic pathway when it was subjected to hydrolysis and a free radical degradation pathway when it was undergoing degradation in the presence of peroxide (Fig. 6). Acidic, basic and photolytic studies gave a common degradation product DP-I.

Oxidative degradation studies gave a major degradation product DP-II; DP-III and DP-IV were formed in smaller quantities. Mechanism of the formation of the degradation products (DP-II, DP-III and DP-IV) is schematically represented (Fig. 6). The presence of carboxylic acid in the drug induces the removal of carbon dioxide molecule through free radical pathway by initiating the reaction of the drug with 3% peroxide [22]. Elimination of ketene is observed, as it forms the amine (DP-III). DP-IV is formed, as methane gets eliminated.

In acid degradation study, carbonyl carbon gets protonated and becomes electrophilic. This leads to the attack of nucleophile (water) and forms the tetrahedral intermediate. Nitrogen being a base abstracts the proton from the positive oxonium ion and leaves

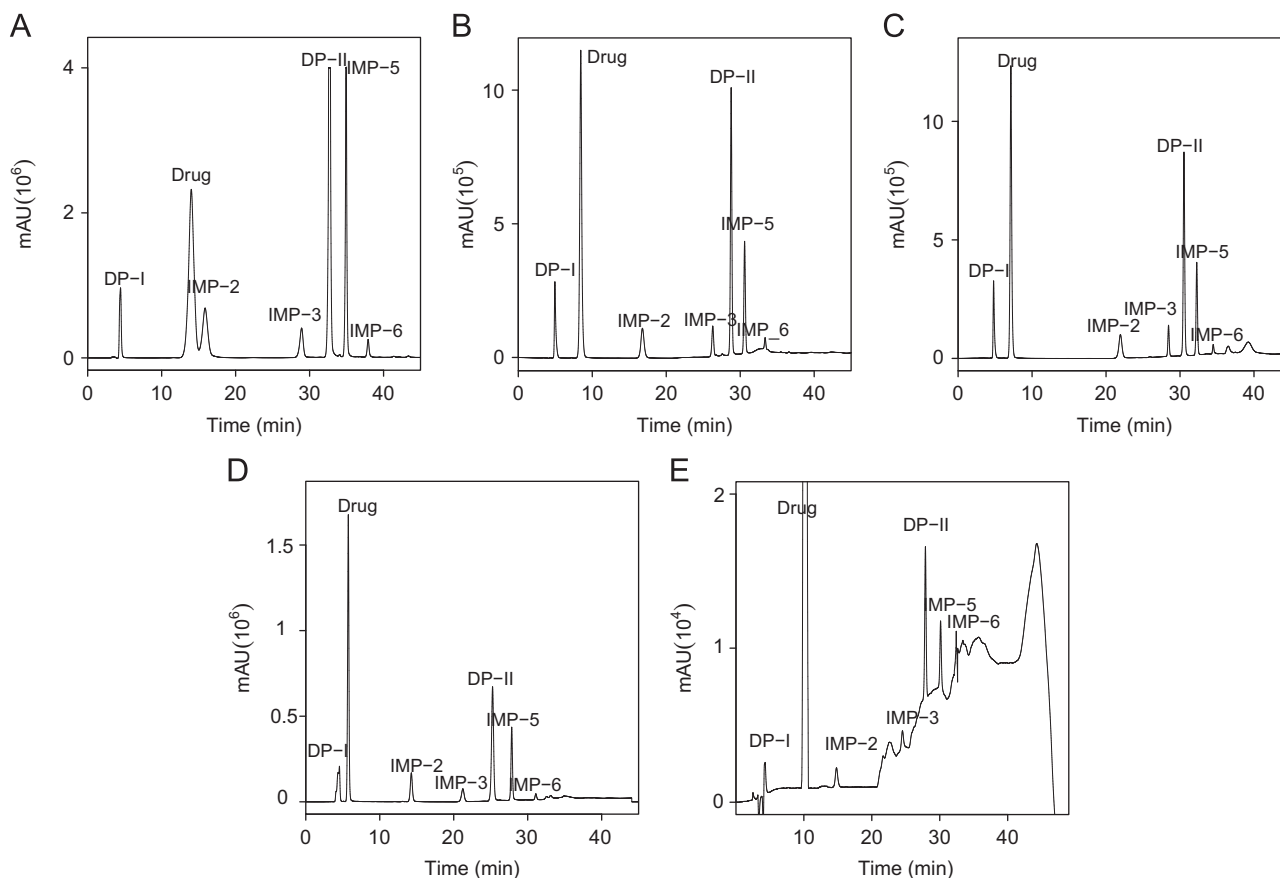


Fig. 4 Chromatograms of the analytes (the drug, impurities and degradation products) under different pH conditions: (A) pH 3.0, (B) pH 5.0, (C) pH 6.0, (D) pH 7.0, and (E) analytes at lower concentrations at pH 5.0.

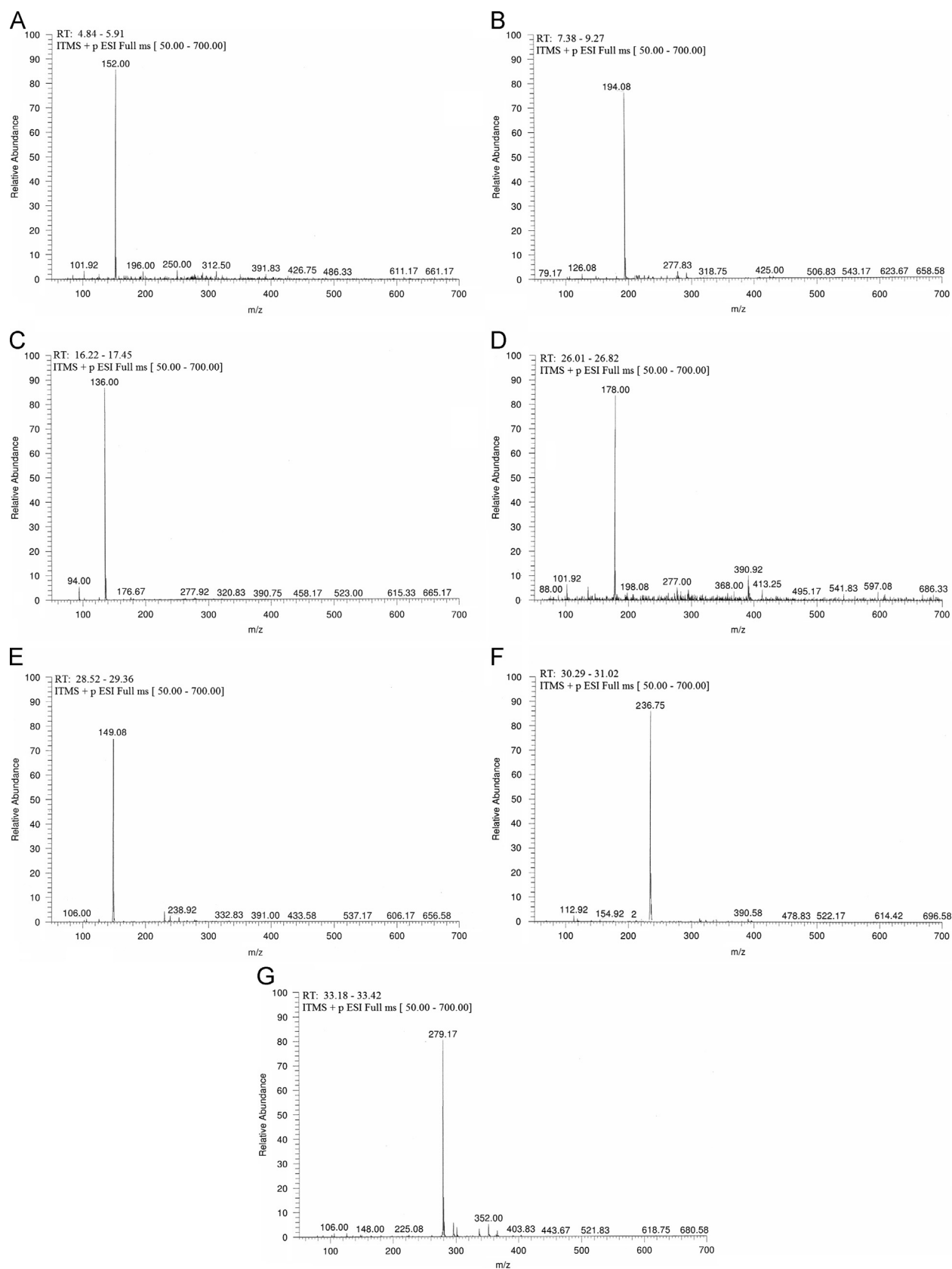


Fig. 5 Mass spectra of the analytes (the drug, impurities and degradation products) taken during the chromatographic run (pH 5.0) in HPLC-PDA-MS: (A) Imp-1; (B) the drug; (C) Imp-2; (D) Imp-3; (E) Imp-4; (F) Imp-5; and (G) Imp-6.

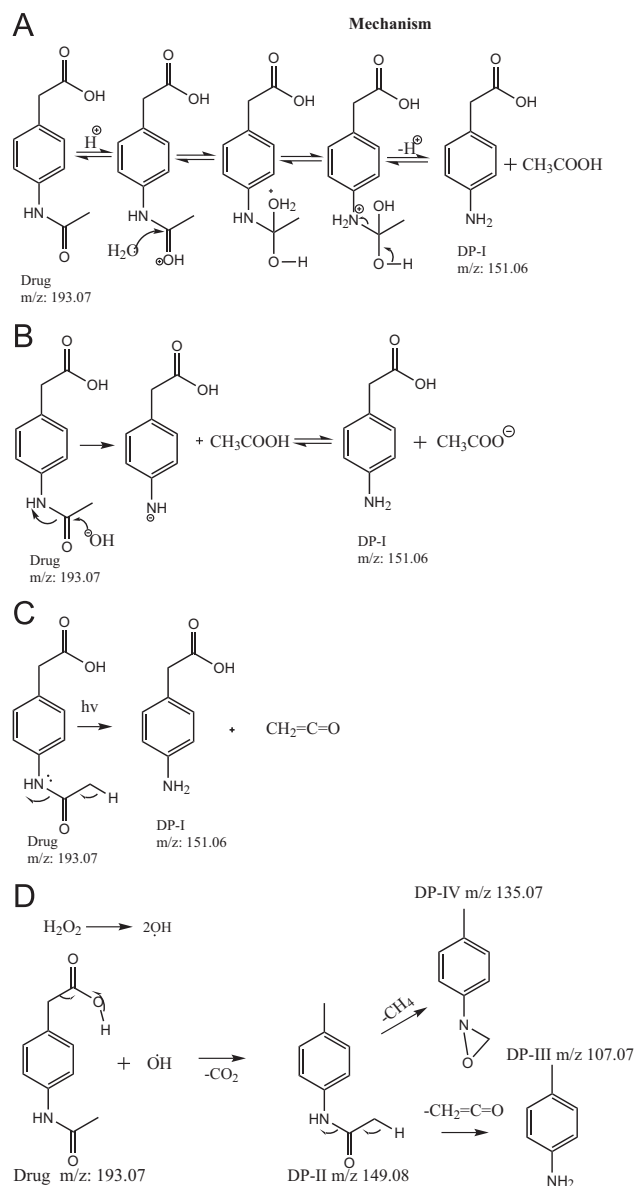


Fig. 6 Mechanism of formation of DP-I: (A) acid hydrolysis; (B) base hydrolysis conditions; (C) photolysis; and (D) mechanism of formation of DP-II, DP-III and DP-IV under oxidative reaction conditions.

as amine (DP-I, m/z 152). Carbocation formed is stabilized by the oxonium ion which then loses a proton to give acid.

In base hydrolysis, hydroxide attacks the electropositive carbonyl carbon, forming the tetrahedral intermediate and leads to the elimination of amide anion. Amide anion abstracts the proton from the acid and forms the neutral amine (DP-I, m/z 152) and carboxylate anion.

In photolytic degradation, amide linkage breaks by free radical mechanism and gives rise to the product DPI (m/z 152). Fragmentation pattern for the drug, degradation products and intermediate was self-explanatory (see Supplementary material).

3.3. Kinetic study

Kinetic studies of actarit (1000 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$) were carried out for three different degradation studies such as acid,

base conditions at three different temperature conditions (60 $^{\circ}\text{C}$, 70 $^{\circ}\text{C}$, and 80 $^{\circ}\text{C}$) for a period of 125 h. The chromatograms indicated that the peak area of the drug decreased with time, revealing that the drug undergone degradation in acidic and basic conditions.

The semilogarithmic plot of concentration of drug against time for 0.1 M HCl/0.1 M NaOH degradation studies gave a straight line, implicating that the degradation pathway has an apparent pseudo first order degradation behavior [23] (see Supplementary material). It was observed that the rate constant of the reaction for both acid and base hydrolysis increased with concentration of the acid, base and also with the concentration of the drug. Increase in temperature enhanced the rate of the reaction in alkaline conditions compared to acidic conditions. The results are tabulated in Table 2. The activation energy in basic and acidic conditions was found to be 11.86 J/mole and 64.45 J/mole, respectively.

3.4. Stability of the drug

The drug was found to be stable in neutral conditions. It was found that stability of the drug increased as the pH increased from 1 to 6. Degradation was found to increase, as the alkalinity increased.

3.5. Method validation

Gradient method which was developed to separate and quantify the analytes (process related impurities, degradation products and the drug) was checked for its efficacy and was validated by the following parameters.

3.5.1. System suitability

System suitability test was used to verify whether the system was adequate for the analysis to be performed; it was an integral part of chromatographic method development. System suitability parameters for the drug and impurities (Imp-1–6) were evaluated and the results are shown in Table 3. Theoretical plates for the impurities and the drug were greater than 2000 [24].

3.5.2. Specificity

The specificity of the developed method was determined by spiking the drug with its six impurities (0.1% with respect to actarit concentration). Specificity of the developed HPLC method was established as the analytes were very well resolved from one another (Fig. 3).

Upslope similarity, down-slope similarity and 3-point peak purity for all the analytes were found to be greater than 0.99 and the peak purity index and single-point threshold for all the analytes were found to be greater than 0.99. This confirms that the method has the ability to unambiguously determine the drug even in the presence of process related impurities and degradation products.

3.5.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of all the impurities namely Imp-1 to -6 were achieved. Precisions at the LOQ concentrations for all the six impurities were below 2.0%. LODs of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 were 0.0797, 0.0384, 0.0746, 0.1119, 0.0566 and 0.0789 $\mu\text{g/mL}$, respectively. LOQs of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 were 0.2658, 0.1282, 0.2487, 0.3732, 0.1889, and 0.2024 $\mu\text{g/mL}$, respectively.

3.5.4. Precision

Precision was determined in terms of intra-day repeatability and inter-day reproducibility. The drug was spiked with 0.1% of the impurities and the degradation products. Intra-day (repeatability) and inter-day (reproducibility) data were determined for impurities 1–6 and the drug. The intermediate precision of the assay method was also evaluated. The results are tabulated in Table 4.

Table 2 Kinetics of the degradation of the drug in acid and base mediated reactions.

Conditions		Rate constants		Half life	
pH	Temperature (°C)	LC ^a	HC ^b	LC ^a	HC ^b
Basic	60	0.01	0.03	46.22	21.00
	70	0.05	0.11	13.86	6.07
	80	0.06	0.13	10.82	5.13
Acidic	60	0.03	0.02	53.30	27.70
	70	0.02	0.03	27.71	18.20
	80	0.03	0.05	19.83	11.71

LC^a: Low concentration of the drug.

HC^b: High concentration of the drug.

Table 3 System suitability parameters for the drug, its process related impurities and degradation products.

Compounds	Capacity factor	Resolution	Tailing factor
DP-I/Imp-1	5.7	0.08	1.87
Imp-2	44.01	12.17	1.27
Imp-3	56.22	5.17	0.79
DP-II/Imp-4	60.40	3.36	1.12
Imp-5	64.22	3.85	1.35
Imp-6	63.92	3.91	1.58
Drug	8.13	5.23	1.39

Table 4 Precision data for the drug, its process related impurities and degradation products.

Experiment	Impurities							Drug	
	Conc. (µg/mL)	RSD (%)						Conc. (µg/mL)	RSD (%)
		Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6		
Repeatability	0.3	1.47	0.46	0.87	0.35	0.85	0.74	300	0.82
	0.5	0.55	0.89	0.57	0.84	0.72	1.38	500	0.45
	0.6	0.72	0.84	0.48	0.59	0.87	0.78	600	0.27
Reproducibility	0.3	0.75	0.69	0.92	0.39	1.30	0.96	300	0.37
	0.5	0.45	0.27	0.84	0.47	1.34	1.47	500	0.21
	0.6	0.77	0.49	0.94	0.84	0.89	0.79	600	0.71
<i>Intermediate precision</i>									
Intra-day recovery	0.3	1.24	1.34	1.28	0.58	1.26	1.02	300	0.79
	0.5	1.38	1.27	1.14	0.72	1.24	1.43	500	0.68
	0.6	0.89	0.78	0.46	0.58	0.85	0.48	600	0.89
Inter-day recovery	0.3	1.26	0.88	1.31	1.24	1.10	1.02	300	0.58
	0.5	0.57	0.81	0.54	0.86	1.24	1.14	500	0.64
	0.6	0.72	0.69	0.85	0.91	0.79	0.69	600	0.74

3.5.5. Robustness

To determine the robustness, three parameters were varied: flow rate, pH and percent composition of the organic modifier. The drug was spiked with process related impurities and degradation products and the resolution among the analytes was monitored. The influence of pH of the mobile phase on the degradation product and the impurities was also studied by analyzing the standard mixture of analytes at three different pH values (Table 5). The effect of mobile phase flow rate on the drug and impurities was evaluated by calculating the resolution factors and the results are tabulated in Table 5.

3.5.6. Linearity

Calibration solutions (drug) were prepared from stock at six different concentration levels from 60% to 120% of the assay analyte concentration (300, 350, 400, 450, 500, and 600 µg/mL). Linearity test solution for the related substance method was prepared by diluting the impurity stock solution to the required concentrations. Six different concentration levels of the solutions (impurities) were prepared in this range (LOQ–0.6 µg/mL).

Calibration studies for assay and purity method were carried out for three consecutive days in the same concentration range. RSD value for the slope and Y-intercept of the calibration curve was calculated. Peak area under the curve (average peak area of the triplicates) was plotted against the respective concentration level (Table 6). Straight lines were obtained and the calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. Y-intercepts obtained for the drug and other analytes were insignificant.

3.5.7. Accuracy

The accuracy of the assay method was evaluated in triplicates at three different concentration levels, i.e. 100, 400, and 600 µg/mL in the bulk drug sample. The drug was spiked with 0.1% impurities and degradation products, and the accuracy of the impurities and degradation products was evaluated. Recovery experiments were conducted to determine the accuracy of the related substance method to quantify the impurities in bulk drug samples. Percentage recoveries were calculated from the slope and

Table 5 Robustness data for the drug, its process related impurities and degradation product.

Parameters	Value	Resolution					
		Drug	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
Flow rate (mL/min)	0.6	2.71	12.63	4.25	10.86	5.81	3.05
	0.8	5.02	17.82	10.94	5.73	4.62	7.55
	1.0	3.75	35.43	3.34	9.62	5.20	4.67
pH	4.9	7.42	21.83	10.01	1.98	4.74	3.73
	5.0	8.99	13.28	15.64	2.01	4.99	6.98
	5.1	8.12	12.12	16.64	1.32	4.22	4.59
Organic modifier	10%	8.99	13.28	15.64	2.01	4.99	6.98
	20%	3.18	20.24	13.26	1.20	1.54	4.32

Table 6 Linearity parameters for the drug, its process related impurities and degradation products.

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	Drug
Trend line equation	$y=47,207x-2254$	$y=13,396x+1020$	$y=62,981x-2518$	$y=81,566x-1462$	$y=91,676x-6563$	$y=14,661x-2272$	$y=117,173x-420,633$
Linearity range ($\mu\text{g/mL}$)	0.2–0.6	0.1–0.6	0.2–0.6	0.3–0.6	0.1–0.6	0.2–0.6	100–600
Regression coefficient	0.9990	0.9991	0.9980	0.9995	0.9992	0.9980	0.9990
Slope	47,207	13,396	62,981	81,566	91,676	14,661	117,173
Intercept	-2254	1020	-2518	-1462	-6563	-2272	-420,633
P-value B	$1.05e-12$	$2.63e-11$	0.9049	0.9985	0.9973	0.029	$2.2e-16$
P-value F	0.58	0.85	0.86	0.93	0.69	0.94	0.58
Chi square	4.69	2.62	2.54	1.88	3.86	1.65	13.23
Sensitivity	46,882	13,376	62,808	81,195	92,292	14,256	117,592

Y-intercept of the calibration curve developed for the drug. Percentage recoveries for the drug and impurities were within the range 96–102%.

4. Conclusion

Synthesis of the drug was carried out taking into consideration the green chemistry. The advantages of the synthetic methodology are (i) simple method, (ii) good yield without using chromatographic techniques, and (iii) environmentally friendly nature as it does not produce toxic by-products. The intrinsic stability of the drug was evaluated under different forced degradation conditions. The developed stability-indicating assay method (SIAM) for the drug, impurities and degradation products was validated as per ICH guidelines. Degradation products that were found in smaller quantities were identified using HPLC–PDA–MS.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jpha.2014.01.002>.

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