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Original Article

Force degradation behavior of glucocorticoid deflazacort by UPLC: isolation, identification and characterization of degradant by FTIR, NMR and mass analysis

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Abstract

In this investigation, sensitive and reproducible methods are described for quantitative determination of deflazacort in the presence of its degradation product. The method was based on high performance liquid chromatography of the drug from its degradation product on reverse phase using Acquity UPLC BEH C18 columns (1.7 µm, 2.1 mm × 150 mm) using acetonitrile and water (40:60 V/V) at a flow rate of 0.2 mL/minute in UPLC. UV detection was performed at 240.1 nm. Deflazacort was subjected to oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The drug was found to be stable in water and thermal stress, as well as under neutral stress conditions. However, forced-degradation study performed on deflazacort showed that the drug degraded under alkaline, acid and photolytic stress. The degradation products were well resolved from the main peak, which proved the stability-indicating power of the method. The developed method was validated as per ICH guidelines with respect to accuracy, linearity, limit of detection, limit of quantification, accuracy, precision and robustness, selectivity and specificity. Apart from the aforementioned, the results of the present study also emphasize the importance of isolation characterization and identification of degradant. Hence, an attempt was made to identify the degradants in deflazacort. One of the degradation products of deflazacort was isolated and identified by the FTIR, NMR and LC-MS study.

Keywords: Deflazacort, forced degradation, degradant, characterization

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Introduction

Deflazacort is an oxazoline prednisolone derivative with the chemical structure of (11β,16β)-21-(acetyloxy)-11-hydroxy-2′-methyl-5′H-pregna-1,4-dieno [17,16-d] oxazole-3, 20-dione[1]. It is a glucocorticoid with potent anti-inflammatory and immunosuppressant activity[2-4]. It is prescribed in the treatment of asthma, arthritis, myasthenia gravis, systemic lupus erythematosus, and thrombocytopenia[5]. Orally administered deflazacort is well absorbed and immediately converted by plasma esterases to the pharmacologically active metabolite (D 21-OH), which achieves peak plasma concentrations in 1.5 to 2 hours[5-6]. Elimination takes place primarily through the kidneys; 70% of the administered dose is excreted through urine and the remaining 30% is eliminated through the feces. Metabolism of D 21-OH is extensive and only 18% of urinary excretion represents D 21-OH[7]. The metabolite of D 21-OH, deflazacort 6-beta-OH represents one-third of urinary elimination[3,6].

Stability is defined as the capacity of a drug substance or a drug product to remain within established specifications to ensure its identity, strength, quality, and purity throughout the retest period or expiration dating period, as per appropriate International Conference on Harmonization (ICH) Q1a/Q1c guidelines[8]. In a typical study, relevant stress conditions like light, heat, humidity, hydrolysis (acid / base influence) and oxidation or even a combination of described parameters are commonly tested[9-10]. If it is necessary to form degradation products, the strength of stress conditions can vary due to the chemical structure of the drug substance, the kind of drug product, and product specific storage requirements. An individual program has to be set up in order to reach a target degradation of 5% to 20%[11-12].

A higher level of degradation will be out of the scope of product stability requirements and therefore unrealistic technically[13]. The scope of the test is to generate degradation products in order to facilitate a method development for determination of the relevant products[14]. Therefore, samples will be stressed in a solid and/or in a solution form. Typically, stress tests are carried out on one batch of material[15]. For drug products, the placebo should be stressed in a similar way in order to exclude those impurities that are not degradation products (e.g. impurities arising from excipients)[16]. Drugs that are poorly soluble in water can be conducted either in suspension or in solution using inert organic co-solvents (e.g., DMSO, acetic acid or propionic acid)[17-19]. It is important to avoid co-solvents that may be reactive with the drug or complicate analysis (e.g. by LC-MS)[15,20].

The ICH guideline entitled "Stability testing of new drug substances and products" requires stress testing to be carried out to elucidate the inherent stability characteristics of the active substance[18,21-23]. Susceptibility to oxidation is one of the required tests. The hydrolytic and photolytic stability tests are also required. An ideal stability indicating method should quantify the standard drug alone and also resolve its degradation products[17,24-25].

There is no official method for analysis of the degradation product of deflazacort in any of the pharmacopoeia. Few liquid-chromatographic methods for the determination of the degradation product of deflazacort have been reported in the literature. Paulino *et al.* also investigated the degradation product of deflazacort and evaluated the anti-inflammatory effect of both deflazacort and its major degradation product[7]. Sharma *et al.* and More *et al.* also investigated the stability of deflazacort in dosage form by HPLC method; however, the analysis was restricted to hydrolysis, photolysis and chemical oxidation[26-27]. Recently, Karthikeyan *et al.* have reported a LC-MS method for estimation of deflazacort products in human plasma[28]. Patel *et al.* reported an ultra-performance liquid chromatography–tandem mass spectrometric method for the determination of 21-hydroxy deflazacort in human plasma using betamethasone as the internal standard[29].

UPLC has various practical advantages, including exponentiality: high sensitivity, accuracy, and resolution apart from the highly decreased analysis time than the reported instruments[30]. As compared to HPLC, UPLC has higher resolution, greater sensitivity and faster analysis time. So UPLC is the more preferred method of sample analysis than HPLC[31]. No method has been reported for performing stability indicating assay of deflazacort by UPLC. The aim of the present work was to develop UPLC based stability indicating chromatographic methods for the determination of deflazacort in the presence of its degradation products.

Materials and methods

Reagents and equipments

An Active Pharmaceutical Ingredient (API) grade of deflazacort was supplied as a gift sample by Wockhardt Pharmaceutical LTD (India). Analytical grades of sodium hydroxide, hydrochloric acid, and anhydrous sodium sulfate, and chromatographic grade acetonitrile and methanol were purchased from Rankem, India. All chemicals and reagents were of HPLC and analytical grade, and were purchased from Merck Chemicals, India. FTIR Spectrophotometer (Shimadzu, FTIR Prestige – 21, USA), LC-MS-MS: 3200 Q trap,

Triple Quadrapole (Applied Biosystem, USA), and Sophisticated multinuclear NMR Spectrometer model Avance-II (Bruker, USA) instrument equipped with cryomagnet of field strength 9.4 T (^{1}H frequency, 400 Mhz; ^{13}C frequency, 100 MHz) were used.

Instrumentation and chromatographic conditions

The Waters Acquity liquid chromatography system (Waters Corporation, USA) with a diode array detector and Acquity UPLC Binary solvent manager pump was used for analytical method development, forced degradation studies and method validation. The output signal was monitored and processed using Empower 2 software (designed by Waters Corporation, USA. The chromatographic column used was an Acquity UPLC BEH C_{18} 150 mm × 2.1 mm, column with 1.7 µm particles. Different mobile phases were tried in order to find the best separation of deflazacort with its degradants. The optimal composition of the mobile phase was determined to be 40:60 V/V acetonitrile:water with retention time of R_t = 5.3 ± 0.01 minutes. Flow rate was set at 0.2 mL/minute, while the injection volume of 2 µL was used for sample injection. The detection was obtained at a wavelength of 240.1 nm.

Standard solution preparation

A stock solution of deflazacort was prepared by dissolving 10 mg in 100 mL acetonitrile (100 µg/mL). The standard solutions were prepared by dilution of the stock solution with acetonitrile to reach a concentration range of 5–25 (µg/mL). For each concentration, 2 µL injection of each standard was made six times in triplicate and chromatographed under the conditions described above. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs (***Supplementary Table 1***).

Mobile phase selection, chromatogram and spectra

Several combinations of different mobile phases were investigated to attain a clean chromatogram (***Table 1***). The mobile phase most suitable for analysis was ACN:Water (40:60) as binary system, a flow rate employed for analysis was 0.2 mL/minute. Chromatogram of deflazacort was observed at 5.324 minute. The complete elution of deflazacort was achieved in 10 minutes at 240.1 nm. Resolution was 4.0–8.0 and tailing, below 1.2 standard chromatogram of deflazacort, is shown in ***Fig. 1*** and ***Supplementary Table 2-12***.

System suitability parameters

The United States Pharmacopoeia (USP) suggests that system suitability tests be performed prior to analysis[32]. The parameters include tailing factor, retention time (R_T), theoretical plate number (N), asymmetry factor, selectivity and % RSD of peak height or area for repetitive injections. Typically, at least 2 of these criteria are required to demonstrate system suitability for the proposed method[33]. Separation variables were set and mobile phase ACN:Water 40:60 V/V was allowed to saturate the column at flow rate 0.2 mL/minute and plotted to get sharp base line. Six replicates of reference standard of deflazacort 10 µg/mL were injected separately. Peak report and column performance report were recorded for all chromatograms.

Table 1 **Mobile phase selection.**

Mobile phase components	Solvent ratio (V/V)	Flow rate (mL/minute)	Retention time in minutes	Remark
			Deflazacort	
MeOH:H_2O	60:40	0.1	4.5	Broad peak
MeOH:H_2O	80:20	0.1	3.6	Not sharp peak
ACN:H_2O	50:50	0.1	3.4	Broad peak
ACN:H_2O	60:40	0.1	3.2	Not suitable
ACN:H_2O	65:35	0.3	2.2	Broad peak and not suitable
ACN:H_2O	70:30	0.2	2.0	RT very less
ACN:H_2O	80:20	0.25	1.8	RT very less
ACN:H_2O	50:50	0.2	4.5	Broad peak and tailing
ACN:H_2O	35:65	0.2	5.6	Not sharp peak
ACN:H_2O	40:60	0.2	5.3	Most suitable

Keywords: RT, room temperature; CAN, acetonitrile. Several combinations of different mobile phases were investigated to attain clean chromatogram. The mobile phase most suitable for analysis was ACN:Water (40:60) as binary system, flow rate employed for analysis was 0.2 mL/minute. The output signal was monitored and processed using Empower 2 software (designed by Waters Corporation, USA. The chromatographic column used was an Acquity UPLC BEH C18 150 mm x 2.1 mm, column with 1.7 µm particles.

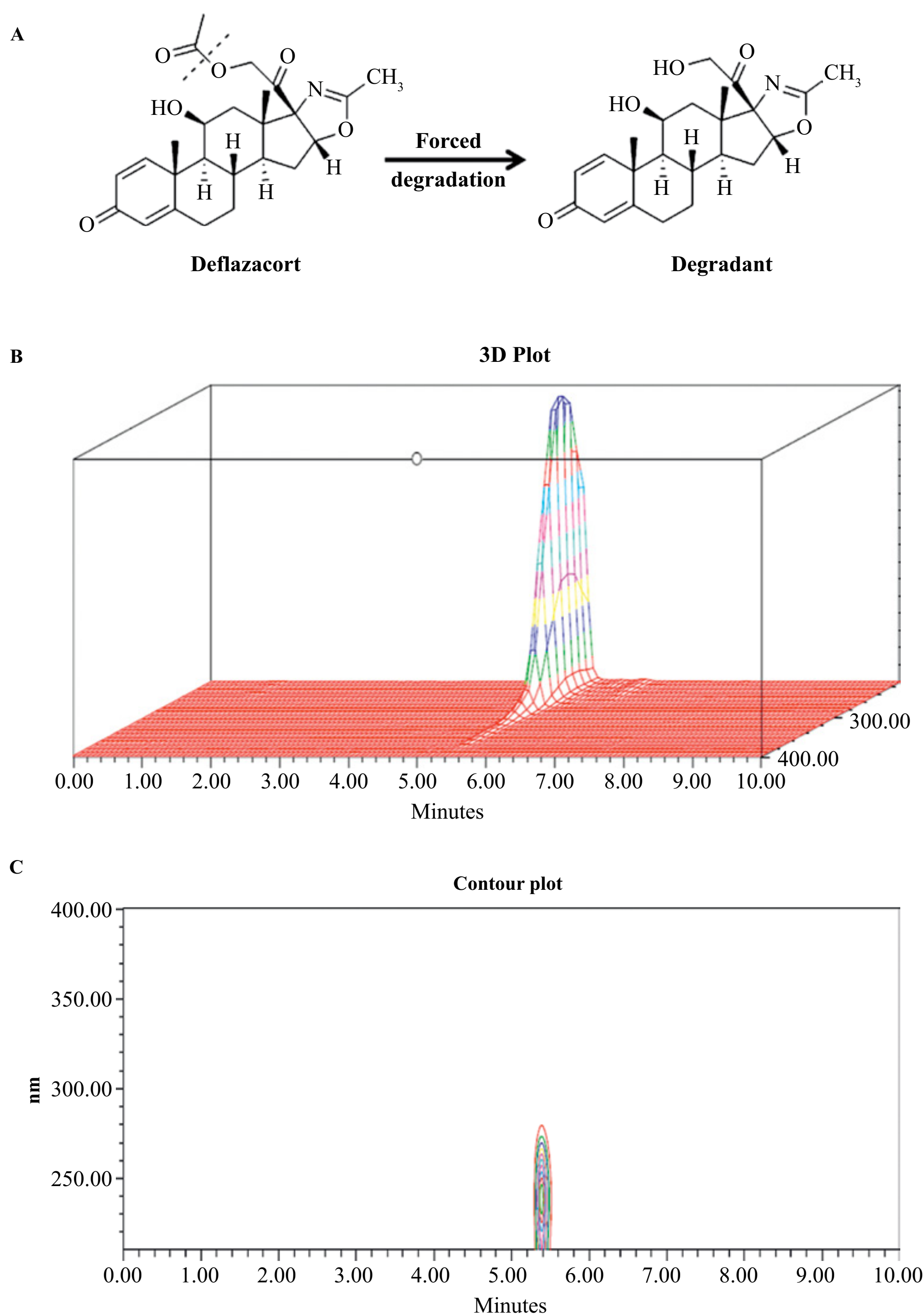

Fig. 1 **Structure of deflazacort and its forced degradation product (A), chromatogram plot of standard deflazacort (B), contour plot of deflazacort (C).** Deflazacort was found to be a stable drug under stress condition such as thermal, neutral and oxidative condition. However, the forced-degradation study on deflazacort showed that the drug degraded under alkaline, acid and photolytic conditions.The name of the identified degradation product is 21 hydroxy deflazocort (11, 21- dihydroxy, 2'-methyl-5'H – pregna-1,4-dione [17,16-d] oxazole- 3, 20-dione.

Development of stability indicating method

Initially, the drug was analyzed on a UPLC BEH column (2.1 × 150 mm × 1.7 um) using acetonitrile and water in the ratio of 40:60 V/V at a flow rate of 0.2 mL/minute and column at room temperature. Under these conditions, the shape of the drug peak and its degradation products were found to be sharp and good at this condition all the degradants and drug was resolved (***Fig. 1***).

Linearity

To establish the linearity, a series of deflazacort dilutions ranging from 5–25 μg/mL were studied individually. All the solutions were filtered through 0.22 μm

Table 2 **Stress studies for hydrolytic degradation under acidic & alkaline condition.**

Category of drug	Strength of acid/alkali	Time of exposure	Temperature	Extent of decomposition
Practically stable	5 N	1 days	Refluxing	None
Very stable	2 N	1 days	Refluxing	Sufficient
Stable	1 N	12 hours	Refluxing	Sufficient
Labile	0.1 N	8 hours	Refluxing	Sufficient
Very labile	0.01 N	8 hours	40°C	Sufficient
Extremely labile	0.01 N	2 hours	20°C	Sufficient

Keywords: N, Normality of solution. Forced degradation studies were carried out to provide stress conditions, that is, acid hydrolysis (0.1 N HCl), base hydrolysis (0.1 N NaOH), oxidation (3 %v/v H2O2), thermal degradation, and photo stability. The peak purity test was carried out for Deflazacort and its degradant peaks by using the PDA detector in stress samples. The isolation of degradant from Deflazacort was performed using flash chromatography (combiflash Rf) having silica redisep Rf 4 gm column. Methanol and chloroform was used as mobile phase in ratio 1:9 at a flow rate 5 mL/minute.

PTFE filter and injected; chromatograms were recorded while the column condition was maintained at room temperature. The solutions were injected into the chromatographic column in triplicate. The linearity of the analytical method was investigated by plotting detector response (the peak area) against analyte concentration. Linear regression analysis was performed to determine slope, intercept and linear correlation coefficient (r^2).

Protocol for separation of drug through flash chromatography

For basic degradation, 100 mg of deflazacort was transferred to a 50 mL volumetric flask and dissolved with 5.0 mL of acetonitrile. The volume was made up with 0.1 N NaOH. The flask was sealed and placed at 37°C in water bath, and then cooled to room temperature. The pH of the solution was adjusted to neutrality by adding 0.1 N HCl. Methanol and chloroform mixture was used as mobile phase in ratio 1:9 at a flow rate of 5 mL/minute.

Forced degradation, isolation and identification of degradant

Forced degradation studies were carried out for deflazacort to provide an indication of the stability indicating property and specificity of the proposed method. Forced degradation studies were also carried out to provide stress conditions including acid hydrolysis (0.1 N HCl), base hydrolysis (0.1 N NaOH), oxidation (3%v/v H_2O_2), thermal degradation, and photo stability (***Table 2-4; Fig. 2, Supplementary Fig. 1-4***). The peak purity test was carried out for deflazacort and its degradant peaks by using the PDA detector in stress samples.

Forced stability studies under stress condition revealed the instability of drug in alkali medium. For isolation of degradant, 100 mg of drug was refluxed with 0.1 N NaOH for 2 hours. The isolation of degradant from deflazacort was performed using Flash Chromatography (Combiflash R_f) having silica Redisep R_f4 gm column. Methanol and chloroform mixture was used as mobile phase in the ratio 1:9 at a flow rate of 5 mL/minute (***Fig. 2-4***). The identification of degradant has been performed using FTIR, NMR and MASS spectroscopy (***Supplementary Table 13, Supplementary Fig. 5-9***). It may be noted that forced degradation studies of deflazacort may be helpful in developing and demonstrating its degradation pathways and degradation products (***Fig. 5***).

Validation of the developed stability indicating method

Intra-day and inter-day precision: Precision and reproducibility of the method were assessed by performing replicate analysis of standard solutions in

Table 3 **Stress studies for hydrolytic degradation under neutral conditions.**

Category of drug	Time of exposure	Temperature	Extent of decomposition
Practically stable	5 days	Refluxing	None
Very stable	2 days	Refluxing	Sufficient
Stable	1 day	Refluxing	Sufficient
Labile	12 hours	Refluxing	Sufficient
Very labile	8 hours	40°C	Sufficient
Extremely labile	2 hours	25°C	Sufficient

Forced degradation studies were carried out to provide stress conditions, that is, acid hydrolysis (0.1 N HCl), base hydrolysis (0.1 N NaOH), oxidation (3 %v/v H2O2), thermal degradation, and photo stability. The peak purity test was carried out for deflazacort and its degradant peaks by using the PDA detector in stress samples. The isolation of degradant from deflazacort was performed using flash chromatography (combiflash Rf) having silica Redisep Rf 4 gm column. Methanol and chloroform was used as mobile phase in ratio 1:9 at a flow rate 5 mL/minute.

Table 4 **Stress studies for degradation under oxidative conditions.**

Category of drug	Strength of hydrogen peroxide	Time of exposure	Temperature	Extent of decomposition
Practically stable	30%	48 days	R.T.	None
Very stable	10%	24 days	R.T.	Sufficient
Stable	3%	24 hours	R.T.	Sufficient
Labile	3%	6 hours	R.T.	Sufficient
Very labile	1%	3 hours	R.T.	Sufficient
Extremely labile	1%	0.5 hours	R.T.	Sufficient

The isolation of degradant from deflazacort was performed using flash chromatography (combiflash Rf) having silica redisep Rf 4 gm column. Methanol and chloroform was used as mobile phase in ratio 1:9 at a flow rate 5 mL/minute.

the mobile phase. Repeatability and reproducibility were characterized for different concentrations and given by mean recovery and relative standard deviation (RSD %). The intra-day precision of the selected method was estimated by the analysis of 5 different concentrations of the drug in triplicate and 3 times on the same day. Inter-day precision was assessed by analyzing samples in the same way as for intra-day precision assay and was repeated for 3 consecutive days[18].

Accuracy: Accuracy was evaluated by fortifying a mixture of degraded solution with five known concentrations of the drug. The recovery of added drug was determined by calculating the pre-analyzed drug concentration and correlating with the concentration of spike drug[17,34].

Robustness: As per ICH guidelines, small, but deliberate variations were made in the method to check the method's capacity to remain unaffected. Robustness was studied at 3 different parameters, viz: change in the ratio of the mobile phase, variation in the flow rate and pH of the mobile phase. Mobile phase ratio of ACN:water varied from 40:60 to 42:58 and 38:62. Changes in the mobile phase concentration are shown in *Supplementary* ***Table 9***. Variation of flow rate was studied from 0.20 mL/minute to 0.19 mL/minute and 0.21 mL/minute (***Supplementary Table 10***).

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

LOD and LOQ are analytical parameters that decide the sensitivity of the developed method. LOQ was taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, whereas LOD was taken as the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified. LOQ was assessed by the standard deviation of the response and the slope method. Slope was calculated from the calibration curve of the analyte and the standard deviation was estimated by running five blank samples while LOD was taken as the one-third of LOQ for their analysis. The LOD was determined by using the signal to noise ratio method.

Specificity and selectivity

Specificity is the ability of the analytical method to measure accurately and specifically the analyte of interest in the presence of other components that might be expected to be present in the sample. The method was found to be specific for the analysis of degradation behavior of deflazacort as well as analysis of the degradant. The PDA analyses proved that the purity-angle value for the drug peak in a mixture of stressed samples was less than purity threshold value, thereby indicating that the drug peak was free from any co-eluting peak. Moreover, the resolution factor for the drug peak was >2 from the nearest resolving peak. Selectivity was assessed by comparing the UPLC traces of standard and stressed deflazacort with degradants.

Results

Mobile phases were investigated serially so as to identify the best separation of pure deflazacort from its degradant products and obtain a clean chromatogram (***Table 1***). ***Fig. 1*** shows a typical chromatogram plot of standard deflazacort. The most suitable mobile phase for analysis of deflazacort and its degradant was found to be ACN and water at a composition of 40:60 V/V binary system and at a flow rate of 0.2 mL/minute (***Supplementary Table 3***). Under these optimized circumstances, a well-separated chromatogram was obtained for deflazacort at a retention time of (5.324 ± 0.01) minutes. The deflazacort, as well as its degradant, showed complete elution from the column in 10 minutes as monitored at 240.1 nm.

The isocratic mode was employed for the elution of deflazacort. Nevertheless, the drugs eluted within 6

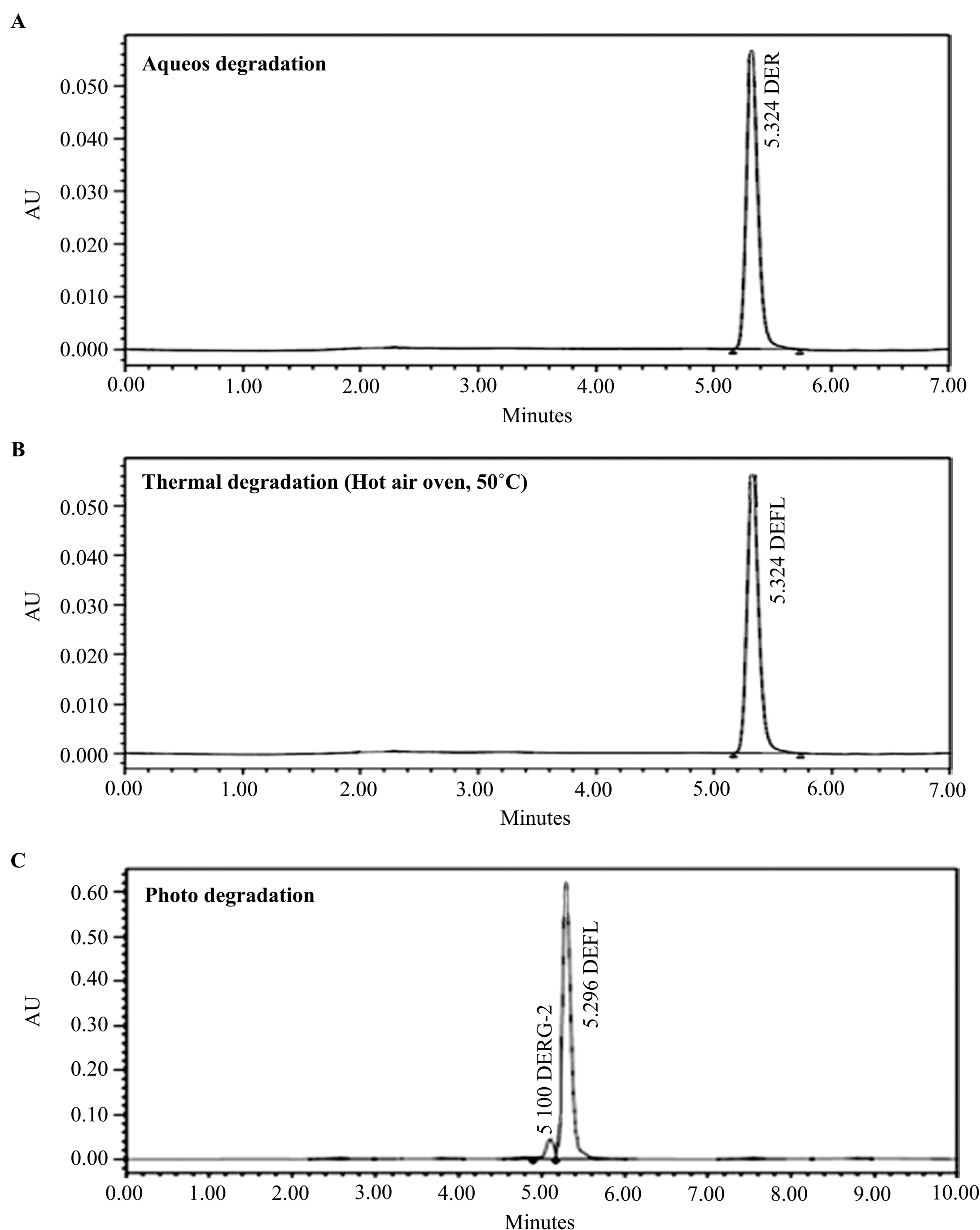

Fig. 2 **UPLC chromatograph of deflazacort and its degradants upon exposure to (A) to water after 24 days, R_T 5.324; thermal degradation in hot air oven at 50°C(B), and photodegradation study after two days exposure of drug to sunlight(C).**

minutes; the run was further continued for additional 4 minutes to ensure complete removal of traces of drugs from the column. ***Fig. 1*** illustrates the complete chromatogram generated over 10 minutes.

Fig. 2-3 represent the typical stability profiles of various deflazacort solutions. UPLC assay on deflazacort under different stress conditions resulted in distinct degradation behavior (***Supplementary Fig. 1-4***). Deflazacort was found to be stable in water and thermal stress, as well as under neutral stress conditions (***Fig. 2***). However, the forced-degradation study performed on deflazacort showed that the drug underwent degradation under alkaline, acid and photolytic stress conditions (***Table 3-4***).

Chromatograms obtained by running 3 concentrations of stock solution were compared with those obtained initially. Degradant-1, degradant-2 and deflazacort showed the purity angle of 0.88, 0.087 and 1.177, respectively. This corresponds to the purity threshold (USP resolution) of 1.151 (2.536), 0.917 (2.244)

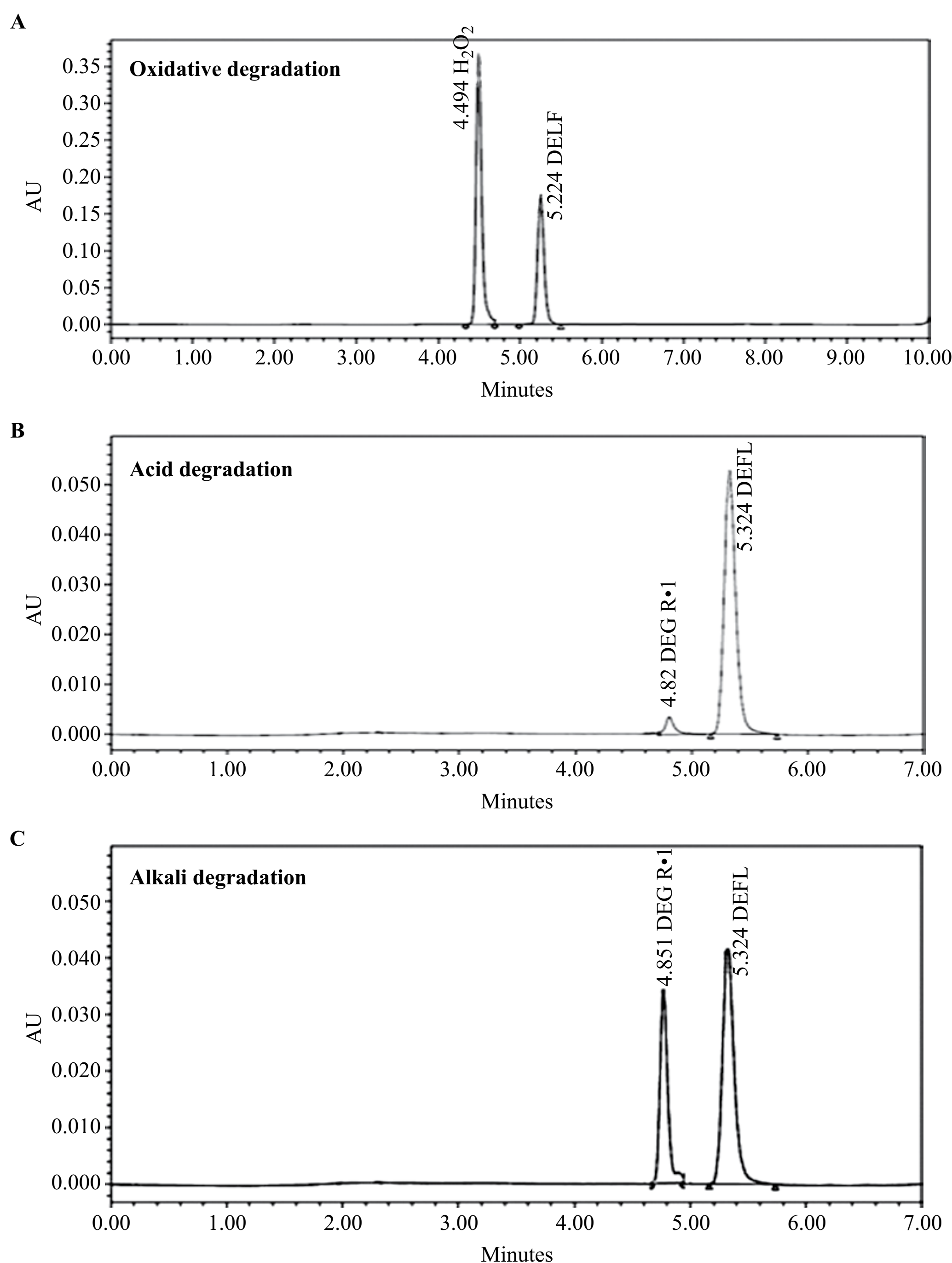

Fig. 3 **UPLC chromatograph of deflazacort and its degradants upon exposure to 30% H_2O_2 for 24 hours, R_T 5.224(A); 0.1N HCl, R_T 5.32(B); 0.1N NaOH(C) after 24 hours of exposure.**

and 2.248 (2.681), respectively, for degradant-1, degradant-2 and deflazacort. Here, the peak area was calculated with respect to the average peak area of the respective concentrations as obtained initially. The linear regression analysis data for the calibration plots show good linear relationship in the concentration range 5–25 µg/mL in UPLC (***Supplementary Table 1***). The method was found to be very sensitive for the determination of pure deflazacort as well as its degradant, with high purity threshold of 1.15, 0.917 and 2.22 for deflazacort, as well as its degradant-1 and degradant-2, respectively.

Degradant product-1 obtained under basic conditions was identified and characterized by FTIR, FTIR and NMR. On the other hand, the degradation product-2 that was obtained in photolytic study was obtained in a very low concentration. The yield of degradant product-2 was very low and did not show a good resolution under flash chromatography. Hence, only degradant product-2 was characterized by FTIR, NMR and MS.

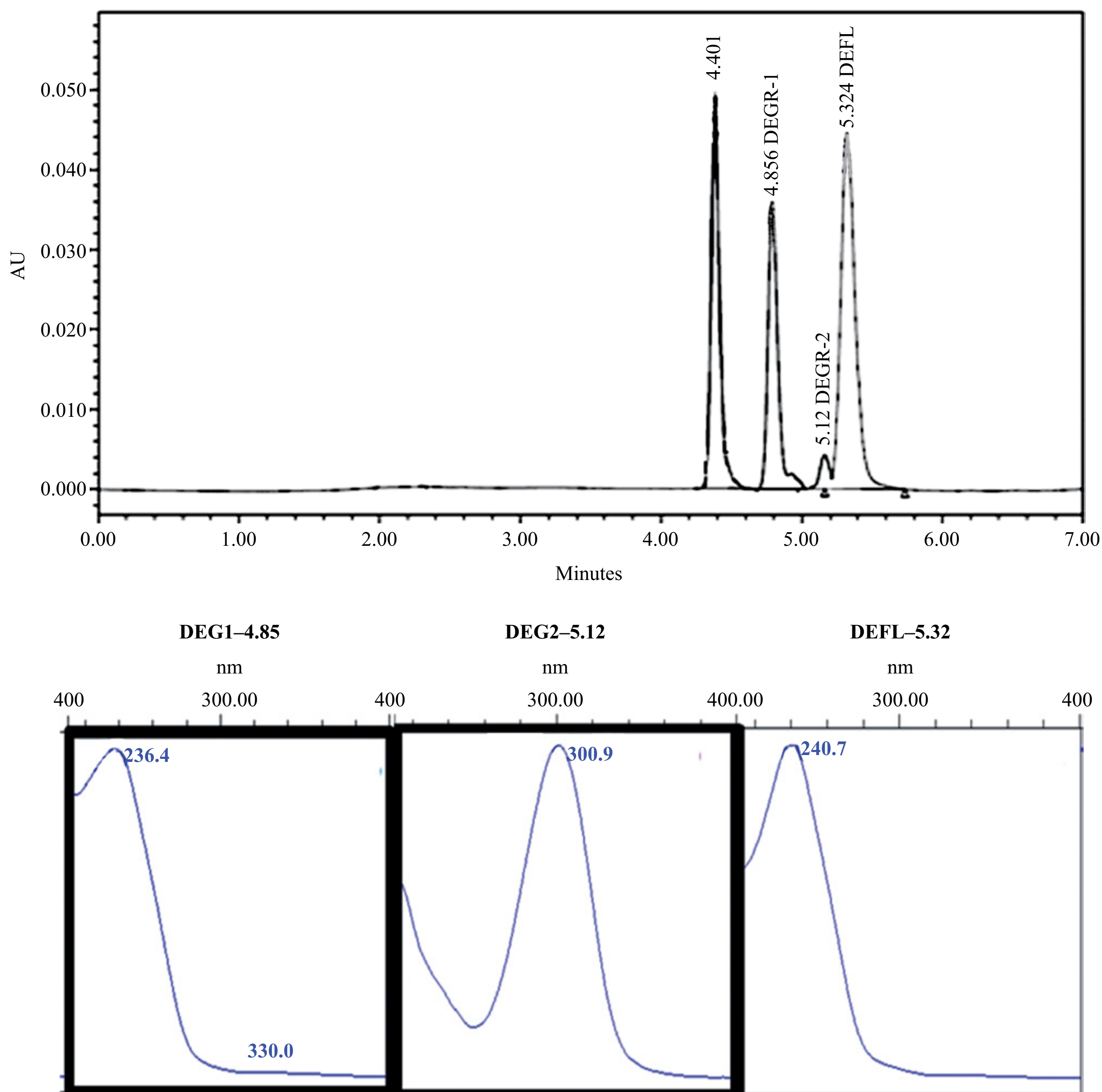

Fig.4 **Method optimization: UPLC chromatogram showing separation of deflazacort and its degradation products in a mixture of stressed samples.**

The degradants were also assayed by FTIR analysis and its typical FTIR peak profile is shown in ***Supplementary Table 13***; ***Supplementary Fig. 5–6***. The ^{1}H NMR (400 MHz, DMSO-d6) **δ** 7.27 (d, J = 10.0 Hz, 1H), 6.12 (dd, J = 10.1, 1.9 Hz, 1H), 5.86 (m, 1H), 5.17 (d, J = 5.3 Hz, 1H), 4.96 (d, J = 6.0 Hz, 1H), 4.70 (d, J = 3.2 Hz, 1H), 4.36 (dd, J = 19.1, 6.0 Hz, 1H), 4.24 (dt, J = 6.5, 3.4 Hz, 1H), 4.14 (dd, J = 19.1, 5.8 Hz, 1H), 2.47 (dt, J = 3.9, 1.9 Hz, 2H), 2.25 (m, 1H of OH), 2.01 (qd, J = 11.1, 6.4 Hz, 2H), 1.92 (m, 2H), 1.83 (s, 3H), 1.66 (m, 1H), 1.34 (s, 3H), 0.82 (s, 3H) (***Supplementary Fig. 9***). The m/z ratio of deflazacort was observed to be 442.8 and, for its degradant product, an m/z ratio of 399.9 (***Supplementary Fig. 7-9***). Based on analytical report analysis, the degradant product-1 of deflazacort was identified as 21 hydroxy deflazocort (11, 21- dihydroxy, 2'-methyl-5'*H* – pregna-1,4-dione [17,16-*d*] oxazole-3, 20-dione. Our literature survey also revealed the presence of one more metabolite of deflazocort, 21-desacetyl deflazacort.

Validation experiments successfully assayed the recovery of 99.86 ± 0.23% deflazacort with % RSD of 0.218. In a similar fashion, the intra-day and inter-day

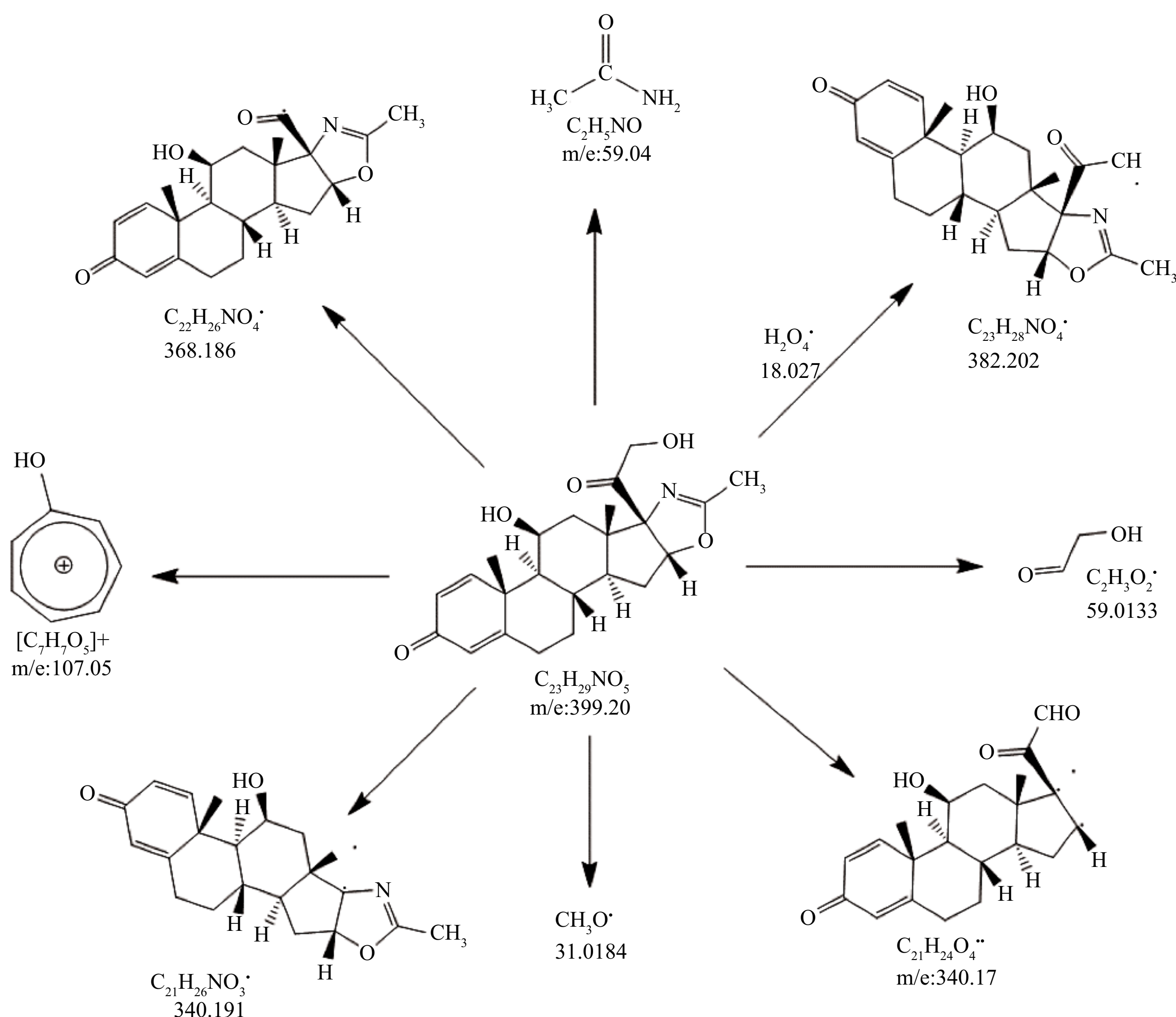

Fig. 5 **Mass fragmentation pathway for degradant product of deflazacort.**

recovery was found to be 99.35 ± 0.40% and 99.53 ± 0.47%, respectively (***Supplementary Table 3***). Precision assay determined the mean recovery of 99.56 ± 0.40 % (0.4 % RSD, ***Supplementary Table 12***).

The concentration that resulted in a signal to noise ratio of 3:1 was found to be 0.05 μg/mL and was determined to be the LOD. A signal to noise ratio of 10:1 was used to determine the LOQ and the concentration elicited a response that could be accurately and reliably measured. This concentration was found to be 0.25 μg/mL. The ruggedness/robustness of the method was checked after deliberately altering the following parameters: composition of the mobile phase, mobile phase flow rate, injection volume, column temperature and detector wavelength (***Table 1***). The parameters of chromatographic separation [retention time, relative retention time (RRT), resolution and number of plates] were not much different on varying the operational parameters.

Validation parameters are highlighted in ***Supplementary Table 9-10*** for deflazacort analysis. The R_T and RR_T of the drug and the degradation products are given in ***Supplementary Table 11***. Purity of the peaks corresponding to the drugs in the method was also established as an additional proof of specificity (***Fig. 1***). Each standard curve was generated in triplicate on 3 consecutive days distributed evenly across the linearity range. Values are reported as mean +SD of 3 calibration curves. Accuracy and precision data showed that the recoveries ranged from 99 to 101%. Both intra- and inter-day precision (% RSD) of QC standards were less than 2% over the selected range for both the drugs (***Supplementary Table 12***).

Triplicate samples were analyzed on 3 consecutive days. For intra-day determinations, 3 standard curves were prepared on the same day. For inter-day determinations, 3 standard curves were generated on 3

consecutive days. Accuracy is represented by percent recovery (mean ± SD) and precision by percent RSD.

Specificity evaluation was carried out by analyzing deflazacort in the presence of its degradant. Both drugs were recovered from solutions and accessed at 5 concentration levels in triplicate (***Table 4***). The results of intentional degradation are summarized in ***Table 3-4***.

Discussion

We have successfully developed a gradient UPLC method for the determination of deflazacort in presence of its degradants. The chromatographic separation method was optimized in Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 150 mm), wherein, different mobile phases were investigated serially so as to find the best separation of pure deflazacort from its degradant products and obtain a clean chromatogram. The most appropriate mobile phase for analysis of deflazacort and its degradant were found to be ACN and water at a composition at 40:60 V/V binary systems, at a flow rate of 0.2 mL/minute. Under these optimized circumstances, a well-separated chromatogram was obtained for deflazacort at a retention time of 5.324 ± 0.01 minutes. The deflazacort, as well as its degradant, showed complete elution from the column in 10 minutes as monitored at 240.1 nm.

UPLC studies on deflazacort under different stress conditions suggested distinct degradation behavior. Deflazacort was found to be stable in water, thermal stress and under neutral stress conditions. However, the forced-degradation study performed on deflazacort showed that the drug degraded under alkaline, acid and photolytic stress. A total of 2 degradation products were observed in samples from stress conditions. The drug purity gradually decreased with time on heating at 80°C in 0.1N HCl, forming degradation product at 4.82 minutes. The rate of hydrolysis in acid was slower when compared to that of alkali. The drug deflazacort found to be very stable to neutral hydrolysis and to be unstable to alkaline hydrolysis. This degradant was isolated from pure drug and the pure degradant, so obtained, was then characterized using LC-MS, FTIR and NMR analyses.

Deflazacort was degraded at 1 hour in 0.1 N NaOH; degradants were obtained at 4.85 minutes and drug peak was obtained at 5.234 minutes. After refluxing the drug for two days, no degradant was obtained and a sharp peak for the drug was obtained at 5.3 minutes. No degradation was observed on exposure of the drug to 30% v/v H_2O_2 for 24 days, showing that it was stable against oxidative stress. The peak of deflazacort was observed at R_T 5.224 minutes. There was no significant degradation of solid deflazacort on exposure to a dry heat at 50°C for 2 months, which indicates the drug was stable against thermal stress.

The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range 5-25 μg/mL in UPLC. Under these conditions, the shape of the peaks for the pure drug and its degradation products were observed to be sharp with high resolution (***Table I***).

System suitability was determined by calculating the %RSD for area and retention time for 6 replicate injections of 10 μg/mL. The % RSD for area under the curve and retention time was found to be 0.003% and 0.400%, respectively in UPLC. All the peaks of degraded product were distinctly resolved from the active pharmaceutical ingredient with significantly different retention times.

The method was validated as per reported guidelines[21,35] and updated international convention[24,36]. The shorter duration of analysis for deflazacort and its degradation products makes the method suitable for routine analysis in pharmaceutical dosage forms. Our validation results showed that excellent recoveries were made at each added concentration, despite the fact that the drug was fortified to a mixture that contained drug as well as the degradation products formed under various stress conditions. By following these optimized chromatographic conditions, there was a higher resolution of chromatographic peak between 4–8 minutes, while low tailing factor was noted below 1.2. The data for intra-day and inter-day precision studies at 3 different concentrations in the linearity range in shown in Table SIX. The % RSD values for intra-day was less than 1%, indicating that the method was sufficiently precise within a day.

The method proves to be specific to each peak, which is indicated through peak purity data, determined through use of a PDA detector. Also, the resolution factor among various peaks is found to be greater than 2, proving good separation between the eluted components. The method was found to be specific, as the drug and degradation products showed good resolution; peak purity data indicate that drug was eluting alone. The LOD and LOQ were found to be 0.05 μg/mL and 0.25 μg/mL, respectively. The specificity and selectivity of the method with the samples under these conditions was demonstrated through the evaluation of retention time (R_T), relative retention time (RR_T), resolution, and purity data for all peaks in the chromatograms.

In conclusion, forced degradation studies of new drug substances and drug products are essential to develop and demonstrate degradation pathways and degradation products of the active ingredients. These studies can be used to guide formulation development and improve manufacturing, as well as packaging processes (temperature, light, acid/alkali treatment, etc.). These

methods are useful to know the degradation pathways of the drug substance in solution, solid state, and drug product.

In this investigation, deflazacort was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Deflazacort was found to degrade significantly in acidic, alkaline and photolytic stress conditions. A simple, reproducible, robust and validated UPLC method was successfully developed to determine degradation products of deflazacort, with a well-resolved degradant peak from main drug peak.

The developed method was validated per ICH guidelines with respect to accuracy, linearity, limit of detection, limit of quantification, precision and robustness, selectivity and specificity. This method shows a huge promise for its application as a stability-indicating assay of deflazacort. The result of this study also emphasizes the importance of isolation characterization and degradant identification. Hence, an attempt was made to identify the degradant in deflazacort. One of the degradation products of deflazacort was successfully isolated and identified by FTIR, NMR, and LC-MS study. The proposed methods are simple, accurate, specific, repeatable, stability indicating, reduce the duration of analysis, and are suitable for routine determination of deflazacort. The method is anticipated to be applied for assessment of purity of bulk deflazacort as well as to predict its stability in dosage forms using UPLC.

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References

[1] Selvadurai M, Meyyanathan SN. Determination of deflazacort in human plasma by liquid chromatography-mass spectrometry after liquid-liquid extraction and its application in human pharmacokinetics studies[J]. *Pharm Methods,* 2011,2(2):106-111.

[2] Walter MC, Reilich P, Thiele S, et al. Treatment of dysferlinopathy with deflazacort: a double-blind, placebo-controlled clinical trial[J]. *Otphanet J Rare Dis*, 2013,8(26).

[3] Saviola G, Abdi Ali L, Shams Eddin S, et al. Compared clinical efficacy and bone metabolic effects of low-dose deflazacort and methyl prednisolone in male inflammatory arthropathies: a 12-month open randomized pilot study[J]. *Rheumatology (Oxford)*, 2007,46(6): 994-8.

[4] Del Rosso A, Cinelli M, Guiducci S, et al. Deflazacort modulates the fibrinolytic pattern and reduces uPA-dependent chemioinvasion and proliferation in rheumatoid arthritis synoviocytes[J]. *Rheumatology (Oxford)*, 2005,44(10):1255-62.

[5] Markham A, Bryson HM. Deflazacort. A review of its pharmacological properties and therapeutic efficacy[J]. *Drugs*, 1995,50(2):317-33.

[6] Rao N, Bhargava VO, Reynolds DL, et al. An investigation of the dose proportionality of deflazacort pharmacokinetics[J]. *Biopharm Drug Dispos*, 1996,17(9): 753-60.

[7] Paulino AS, Rauber G, Deobald AM, et al. Isolation and characterization of a degradation product of deflazacort[J]. *Pharmazie*, 2012,67(6):495-9.

[8] Jain PS. Stability-indicating HPTLC determination of ambroxol hydrochloride in bulk drug and pharmaceutical dosage form[J]. *J Chromatogr Sci*, 2010,48(1):45-8.

[9] Bardin C, Astier A, Vulto A, et al. Guidelines for the practical stability studies of anticancer drugs: a European consensus conference[J]. *Ann Pharm Fr*, 2011,69(4):221-31.

[10] Elragehy NA, Abdel-Moety EM, Hassan NY, et al. Stability-indicating determination of meropenem in presence of its degradation product[J]. *Talanta*, 2008,77(1): 28-36.

[11] Li N, Feng F, Yang B, et al. Simultaneous determination of beta-lactam antibiotics and beta-lactamase inhibitors in bovine milk by ultra performance liquid chromatography-tandem mass spectrometry[J]. *J Chromatogr B Analyt Technol Biomed Life Sci,* 2013,945-946C(110-114).

[12] Baertschi SW, Alsante KM, Tonnesen HH. A critical assessment of the ICH guideline on photostability testing of new drug substances and products (Q1B): Recommendation for revision[J]. *J Pharm Sci*, 2010, 99(7):2934-40.

[13] Jain D, Basniwal PK. Forced degradation and impurity profiling: Recent trends in analytical perspectives[J]. *J Pharm Biomed Anal*, 2013, 86(0):11-35.

[14] Blessy M, Patel RD, Prajapati PN, et al. Development of forced degradation and stability indicating studies of drugs—A review[J]. *J Pharm Anal*, 2014,4(3):159-165.

[15] Jain D, Basniwal PK. Forced degradation and impurity profiling: recent trends in analytical perspectives[J]. *J Pharm Biomed Anal*, 2013, 86(11-35).

[16] Traple MAL, Saviano AM, Francisco FL, et al. Measurement uncertainty in pharmaceutical analysis and its application[J]. *J Pharm Anal*, 2014,4(1):1-5.

[17] Tekade RK, Dutta T, Jain N, et al. Simultaneous Spectrophotometric Estimation of Methotrexate and All-trans Retinoic Acid in Mixture[J]. *Asian J Chem*, 2009, 21(7):5145-5150.

[18] Tekade RK, D'Emanuele A, Elhissi A, et al. Extraction and RP-HPLC determination of taxol in rat plasma, cell culture and quality control samples[J]. *J Biomed Res*, 2013,27(5): 394-405.

[19] Thakur S TRK, P.; Jain N.K. The effect of polyethylene glycol spacer chain length on the tumor targeting potential of folate modified PPI dendrimers[J]. *J Nanopart Res,* 2013,15(1625).

[20] Stanisz B, Regulska K. Kinetics of degradation of imidapril hydrochloride in finished dosage formulations[J]. *Acta Pol Pharm*, 2013,70(4):737-42.

[21] International Conference on Harmonisation; evaluation of stability data; availability. Notice[J]. *Federal register*, 2004,69(110):32010-1.

[22] Maheshwari RG, Tekade RK, Sharma PA, et al. Ethosomes and ultradeformable liposomes for transdermal delivery of clotrimazole: A comparative assessment[J]. *Saudi Pharm J*, 2012,20(2):161-70.

[23] Tekade RK, Gajbhiye V, Rai K, et al. Development of colorimetric method for determination of nitrazepam in tablets and bulks[J]. *Int J Pharm Pharm Sci*, 2010,1(47-52).
[24] International Conference on Harmonisation; Stability Data Package for Registration Applications in Climatic Zones III and IV; Stability Testing of New Drug Substances and Products; availability. Notice[J]. *Federal register*, 2003, 68(225):65717-8.
[25] Vukkum P, Deshpande GR, Babu JM, et al. Stress Degradation Behavior of Abacavir Sulfate and Development of a Suitable Stability-Indicating UHPLC Method for the Determination of Abacavir, its Related Substances, and Degradation Products[J]. *Sci Pharm*, 2012,80(4):903-21.
[26] Shweta W. More SSY, Sheetal S. Shinde, Janhvi R. Rao. Development and validation of stability-indicating RPHPLC method for estimation of deflazacort in pharmaceutical dosage form[J]. *Int J Pharm Pharm Sci,* 2013,5(4):166-171.
[27] Yogesh Sharma NJ, Harshita Jain, Prateek Kumar Jain and Ashish Kumar Jain. Development and validation of RP-HPLC method of deflazacort for stability indicating assay[J]. *Int J Pharm Sci*, 2014,4(2): 117-123.
[28] Karthikeyan S, Aji A, Singh S, et al. A LC/MS method for the quantification of deflazacort metabolite in human plasma: development, validation and application to a pharmacokinetic study[J]. *J drug deliv ther*, 2013,3(2):75-82.
[29] Patel DP, Sharma P, Patel BM, et al. Analysis of 21-hydroxy deflazacort in human plasma by UPLC–MS/MS: Application to a bioequivalence study in healthy volunteers[J]. *J Pharm Biomed Anal*, 2013,85(0): 213-217.
[30] Lee SM, Jeong HJ, Chang IS. Determination and validation of six sunscreen agents in suncare products by UPLC and HPLC[J]. *J Cosmet Sci*, 2008,59(6): 469-80.
[31] Novakova L, Solichova D, Solich P. Advantages of ultra performance liquid chromatography over high-performance liquid chromatography: comparison of different analytical approaches during analysis of diclofenac gel[J]. *J Sep Sci*, 2006,29(16):2433-43.
[32] Li YG, Chen M, Chou GX, et al. Ruggedness/robustness evaluation and system suitability test on United States Pharmacopoeia XXVI assay ginsenosides in Asian and American ginseng by high-performance liquid chromatography[J]. *J Pharm Biomed Anal*, 2004,35(5):1083-91.
[33] Briscoe CJ, Stiles MR, Hage DS. System suitability in bioanalytical LC/MS/MS[J]. *J Pharm Biomed Anal*, 2007,44(2):484-91.
[34] Kesarwani P, Tekade RK, Jain N. Spectrophotometric estimation of paclitaxel[J]. *Int J Adv Pharm Sci*, 2011, 2(1).
[35] Coirini H, Flores D, Vega MC, et al. Binding of the anti-inflammatory steroid deflazacort to glucocorticoid receptors in brain and peripheral tissues. *In vivo* and in vitro studies[J]. *J Steroid Biochem Mol Biol*, 1994,49(1):43-9.
[36] International Conference on Harmonisation: guidance on Q1D bracketing and matrixing designs for stability testing of new drug substances and products; availability. Notice[J]. *Federal register*, 2003,68(11):2339-40.

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