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Chromatography method transfers from HPLC to a new generation instrument UPLC and studies on force degradation behavior of deflazacort

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ABSTRACT

Study describes the development of an efficient, cost-effective, rapid and sensitive method of analysis of deflazacort, with a new generation equipment ultra performance liquid chromatography with photo diode array detector as per international conference on harmonization guidelines. The analytical method validation was compared with high performance liquid chromatography with ultra violate detector. Chromatography was carried out on an UPLC BEH@C18 column (50×2.1 mm, particle size 1.7 µm) whereas for high performance liquid chromatography with ultra violate detector; analysis done on Sunfire C18 column (150x4.6 mm, particle size 5 µm) was used. The mobile phase for ultra performance liquid chromatography consisted of methanol: water (70: 30 v/v) with a flow rate of 0.25 mL/min, whereas for high performance liquid chromatography it was 80: 20 v/v and flow rate 1.0 mL/min respectively was taken. The detection was achieved at 240 nm for both instruments. The stability indicating method was confirmed by various stress studies like acidic, alkaline, oxidative, photolytic and thermal as per international conference on harmonization recommendations. The analytical method validation was performed on ultra performance liquid chromatography and all parameters e.g. solution stability, system suitability, accuracy, precision, linearity and range, robustness, limit of detection and limit of quantification and forced degradation study met the acceptance criteria as per international conference on harmonization guidelines. The degradation products were well separated in ultra performance liquid chromatography. Additionally, the major degradation product of deflazacort was identified by liquid chromatography-electron spray ionization coupled with mass detector.

Keywords: Deflazacort, UPLC-PDA, HPLC-UV, LC-ESI-MS, Degradation products

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INTRODUCTION

Deflazacort is an oxazoline analogue of prednisolone with anti-inflammatory and immunosuppressive properties [1]. Its chemical name is (11β, 16β)-21-(acetyloxy)-11-hydroxy-2'-methyl-5'-pregna-1, 4-dieno {17, 16D} oxazole-3, 20-dione [2]. This dual indicative glucocorticoid drug is also given to patients suffering from rheumatoid arthritis, asthma and other indications such as myasthenia gravis, systemic lupus erythematosus, thrombocytopenic purpura, Duchenne muscular dystrophy and kidney transplant patients [3, 4]. This corticosteroid has lower risk with minimum side effects. Some of the well known side effects of such drugs are bone loss, glucose intolerance or Cushing's syndrome etc [5]. Deflazacort is marketed as oral suspension and tablet.

Literature survey reveals that some spectrophotometric and high performance liquid chromatographic assay methods are reported for the determination of deflazacort in different formulations and in body fluid [6-9]. Some analytical techniques like HPTLC and LC-MS are used for the quantization of deflazacort and its metabolite 21-hydroxy-DF

are reported [10-12]. The determination of deflazacort and their metabolites in biological fluids as well as dissolution studies by HPLC are reported [13-15].

The recently introduced UPLC has significant advantages in speed, resolution, sensitivity, time saving and less solvent consumption, which makes it as a highly efficient and cost-effective technique for rapid analysis in a quality control lab. Therefore, with a view to reducing cost of analysis and minimizing run time, vis-à-vis conventional HPLC, a UPLC method was developed for the analysis of deflazacort. The study also reports comparative data with respect to HPLC method and method transfer to UPLC. Additionally, the new degradation product (DP-2, acid degradation product) [16] has been identified by LC-ESI-MS. The one of the stress degradation product 21-Hydroxy deflazacort is potent molecule and have similar biological activity [17]. This study reports development of novel method of analysis of deflazacort with stress degradation study and its validation as per ICH guidelines [18].

MATERIALS AND METHODS

The Deflazacort (API) was gifted by Hetero Labs limited, Visakhapatnam, India. The commercially available deflazacort tablet formulation labeled 6 mg content was obtained from market. HPLC grade methanol was obtained from Merck India Limited, Mumbai, India. High purity de-ionized water was prepared using Milli-Q, Millipore (Milford, USA) water purification system. The other analytical grade chemicals like hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution 30% (v/v) were purchased from Ranbaxy Fine Chemicals (New Delhi, India) whereas 0.45 µm membrane filters were procured from Pall Life Sciences (Mumbai, India).

Preparation of stock and standard solutions:
A deflazacort stock solution (500 µg/mL) was prepared by dissolving 50 mg accurately weighed reference compound in 100 mL volumetric flask with mobile phase. The standard solution (50 µg/mL) was prepared by transferring 5 mL stock solution to 50 mL volumetric flask with the mobile phase.

Preparation of sample solutions:
Twenty tablets were accurately weighed; average weight of tablet was determined and powdered. The mass of tablet (equivalent to 50 mg of deflazacort) was transferred to 100 ml volumetric flask and dissolved in 50 ml mobile phase. The prepared solution was sonicated for 30 min, filtered with 0.45 µm membrane filter then mobile phase was added up to the mark. The 5 mL solution was transferred to 50 mL volumetric flask and diluted up to mark to obtain a solution of approx 50 µg/mL concentration.

Instrumentation:
HPLC-UV:
High performance liquid chromatography was performed with Waters equipment 600 quaternary pump, Waters 2489 UV/Vis detector, Waters 600 controller, Waters in-line degasser AF and manual injector with 20 µL loop. The equipment was connected to a multi-instrument data-acquisition and data-processing system (Empower software).

UPLC-PDA:
Similarly, Waters Acquity UPLC^{TM} System (Switzerland) comprised of a binary solvent manager, a sample manager, PDA detector and Empower 2.0 version software for data acquisition was also used.

LC-ESI-MS:
The LC-ESI- MS method was performed on a Shimadzu LC system (Shimadzu) equipped with a CBM-20A system controller, a binary gradient LC-20AD Pump, a SIL-20ac auto sampler, a CTO-20AC column oven and SPD-M20A PDA detector. Lab Solution software was used for the data acquisition and analysis. The Sartorius microbalance was used for the weighing purpose.

Chromatographic conditions:
A Waters Acquity UPLC @ BEH C18 column with 50x4.0 mm ID and 1.7µm particle size and Sunfire C18 column with 150x4.6 mm, particle size 5 µm were used to achieve the best separation on UPLC and HPLC. The mobile phase consisted of methanol: water (70: 30, v/v) and (80: 20, v/v), used for the separation at flow rate of 0.25 mL/min and 1.0 ml/min, while the injection volume for UPLC and HPLC was 5 µL and 20 µL respectively. To get the best result for LC-ESI-MS, flow rate was optimized up to 0.5 mL/min. The other chromatographic parameters used in the HPLC-UV analysis were used for the LC-ESI-MS analysis also. Based on the absorption maxima observed for the component, the detection wavelength was set at 240 nm. Ultrasonic bath (Spinco Ltd) was used for the mobile phase and sample degassing.

First of all, the mass spectrometer conditions were optimized with a direct injection (2µg/mL) of deflazacort reference standard solution. The mass spectrometer parameter conditions e.g. CID gas, conversion dynode, interface volt and DUIS Corona needle volt were 230 kPa, -6.00 kV, 4.50 kV and 4.50 kV respectively. The mass interface parameters e.g. interface temperature, DL temperature; heat block temperature and nebulizing gas flow were 350^{o}, 300^{o}, 450^{o} and 3 L/min respectively. Degradation samples were injected and full scan spectra were acquired between the range of 10.0 to 1000 m/z.

RESULTS AND DISCUSSION

Method development:

The column selection is the most important part in method development to achieve maximum sensitivity, selectivity and speed. After performing several trials on different chemistry columns e.g. HSS T3 and BEH Phenyl, the maximum separation was achieved on BEH C18 column for UPLC whereas Sunfire C18 column was selected for HPLC. The different mobile phase composition like water: acetonitrile and water: methanol with their different ratio and pH of mobile phases were explored with a view to examining compatibility of the method as per ICH guidelines and finally the solvent system of isocratic elution of methanol: water (70:30 v/v) was chosen for UPLC whereas (80:20 v/v) ratio of mobile was used for HPLC. The chromatograms of standard by HPLC and UPLC are given in fig.1 (a) and (b) respectively.

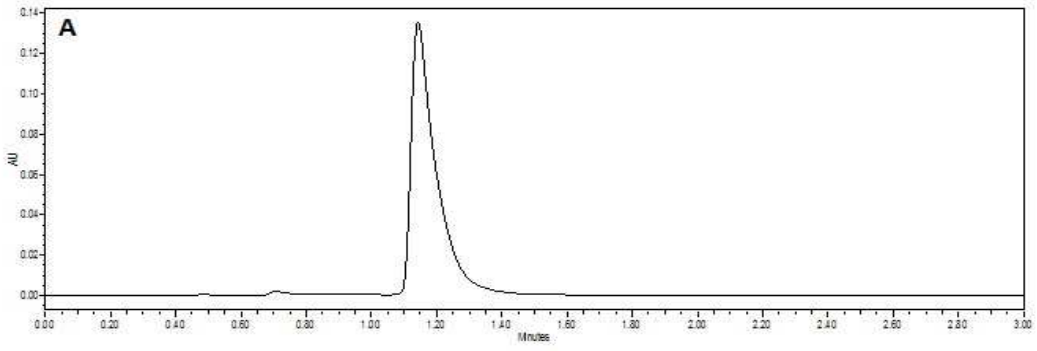

Fig. 1. (a) UPLC Chromatogram of standard Deflazacort

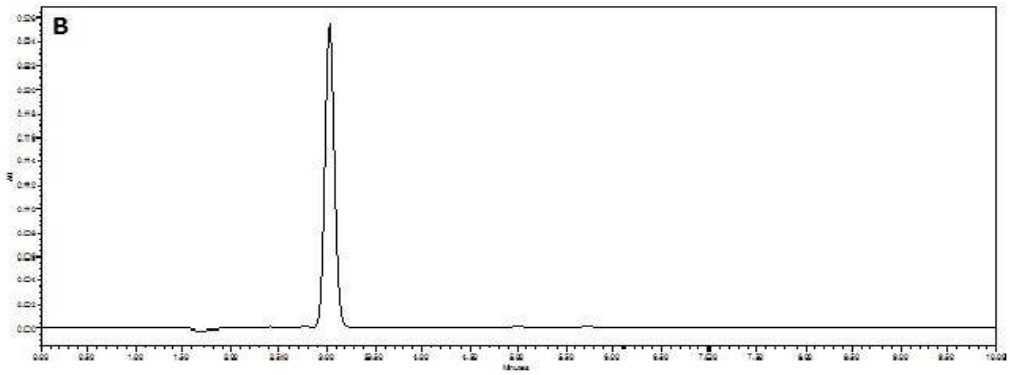

Fig. 1. (b) HPLC Chromatogram of standard Deflazacort

Method transfer from HPLC-UV to UPLC-PDA:

As the term technology transfer suggests that the earlier developed and validated stability-indicating HPLC method for the determination of deflazacort was optimized to achieve the more speed, sensitivity and resolution. The conventional HPLC method was scale down to attain better chromatographic compatibility in order to using smaller particle size column and a new generation UPLC equipment.

Forced degradation study:

To perform forced degradation study, the solutions were subjected to acidic, alkaline and oxidizing condition for thermal and photolytic stress study direct powdered compound was exposed to heat (kept in oven) and sunlight. The chromatographs of alkali, acid and oxidation degradation are given in fig. 2 (a), (b), and (c).

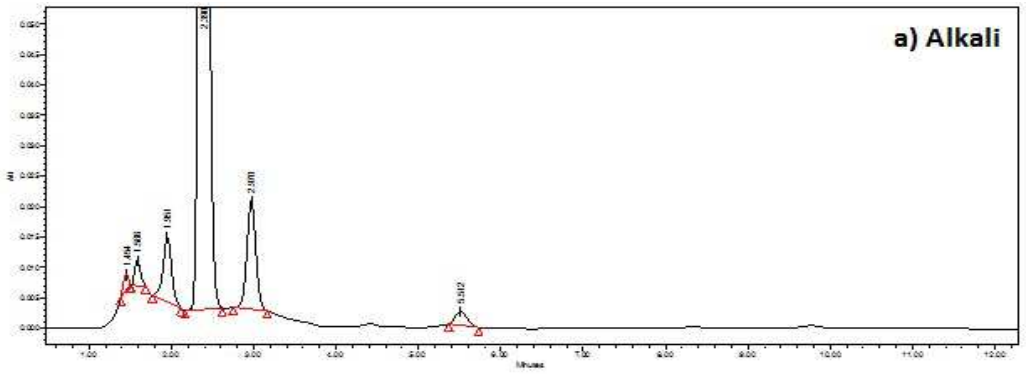

Fig. 2. Chromatographs of Stress degradation study (a) Alkali degradation

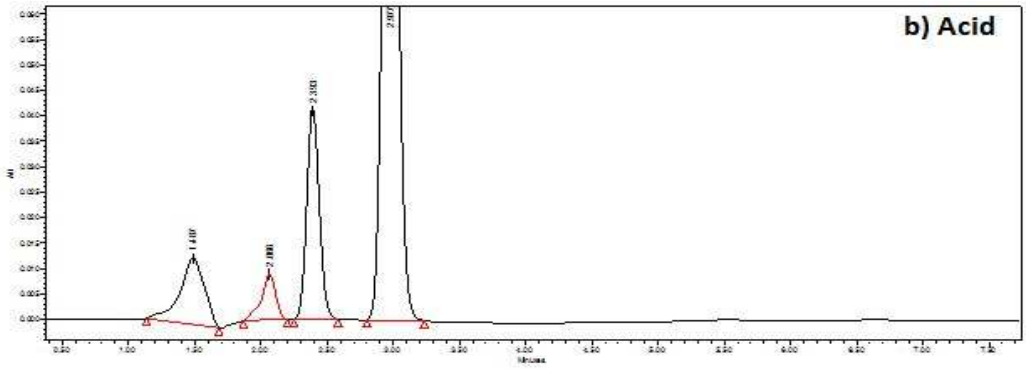

Fig. 2. Chromatographs of Stress degradation study (b) Acid degradation

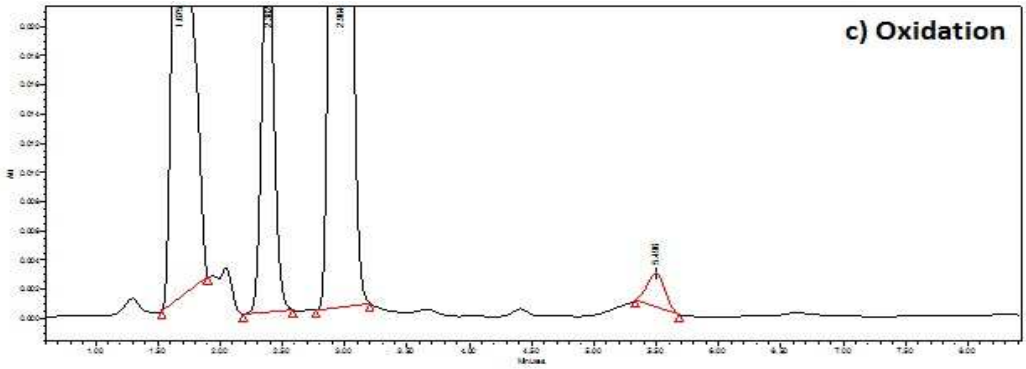

Fig. 2. Chromatographs of Stress degradation study (c) Oxidation degradation

In acidic degradation, the drug was subjected to 0.1 N HCl at room temperature for 6 h and the mixture was neutralized with 0.1 N NaOH solutions. For alkaline stress study, the solution was treated with 0.1 N NaOH at room temperature for 2 h and the mixture was neutralized with 0.1 N HCl. For degradation under oxidizing condition, 3% H_2O_2 solutions was added to stock solution and kept at room temperature for 12 h. For thermal and photolytic degradation, the powdered drug was exposed at 70^o for 6 h in oven and in sunlight for 12 h. After completion of the treatment, the solution were left to room temperature and diluted with mobile phase to furnish approx 500 µg/mL solutions. Further, sample was diluted to obtain 50 µg/mL concentrations. The purity of the drug peak obtained from the stressed sample was measured using PDA detector. The percentage degradation is calculated with respect to peak area of standard solution as summarized in tab. 1.

Tab. 1. Results for stress degradation study by HPLC

Stress Conditions	Drug recovered	Drug decomposed
Standard Drug	100.00 %	--------
Acidic (0.1 M HCl, RT, 6 h)	80.94%	19.06%
Alkaline (0.1 M NaOH, RT, 2 h)	78.85%	21.15%
Oxidative (3% H_2O_2, RT, 12 h)	85.72%	14.28%
Thermal (Oven, 70^o, 6 h)	87.99%	12.01%
Photolytic (Sunlight, 12 h)	94.59%	05.41%

Identification of degradation product by LC-ESI-MS:

In the primary stage of the force degradation study, we have observed the instability of deflazacort under acidic and basic conditions using the LC methods developed for the quantification of deflazacort. In order to propose probable structure of degradation product, LC-ESI-MS measurements were performed. In LC-MS method, retention time of the deflazacort was 2.9 min and retention time of one of the major degradation products for alkali, acidic and oxidative conditions eluted before deflazacort was 2.3 min. The molecular ion peaks obtain from LC-MS analysis in positive scanning mode were m/z 400.6 (mass +H), and m/z 417.22 (mass 418.60 +H) for DP-1 (2.39 min) and DP-2 (1.95 min) respectively. Moreover, for the deflazacort, it obtained at m/z 442.6 (DFZ, mass 441+H) in positive scanning mode. It shows that the degradation product is 21-hydroxy deflazacort (structure is given in fig. 3). LC-ESI-MS results for acid, alkali degradation products and deflazacort are shown in fig. 4.

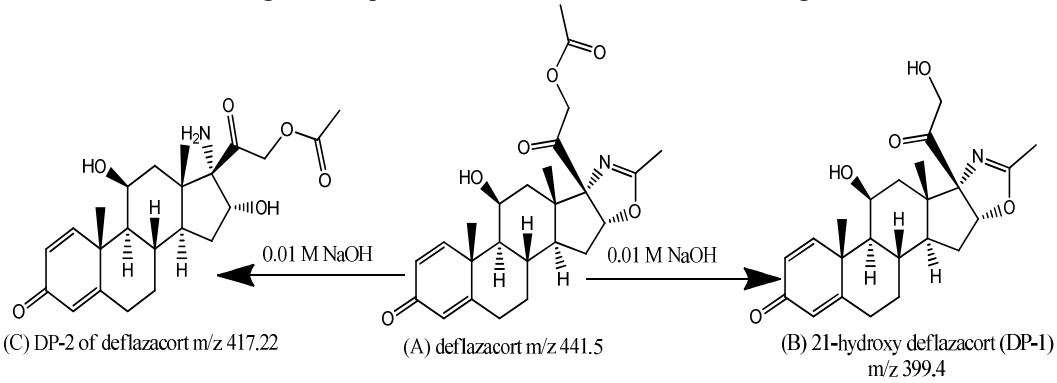

Fig. 3. Structures of deflazacort and proposed degradation products

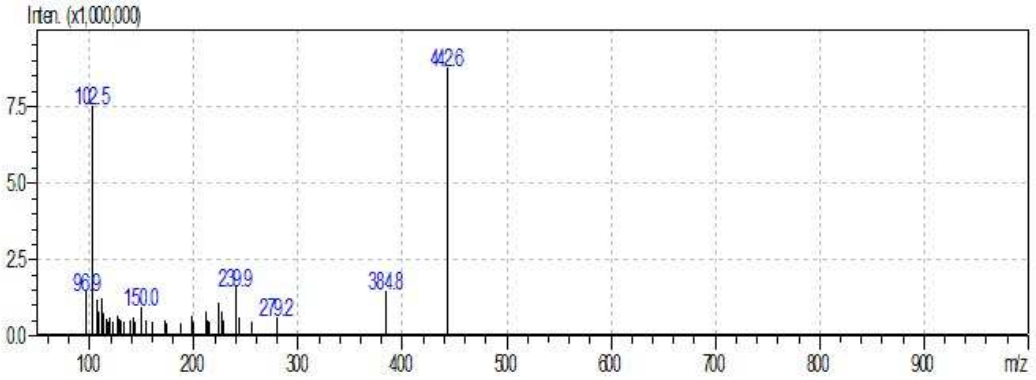

Fig. 4. LC-MS results of degradation products (a) Deflazacort 442.6 m/z

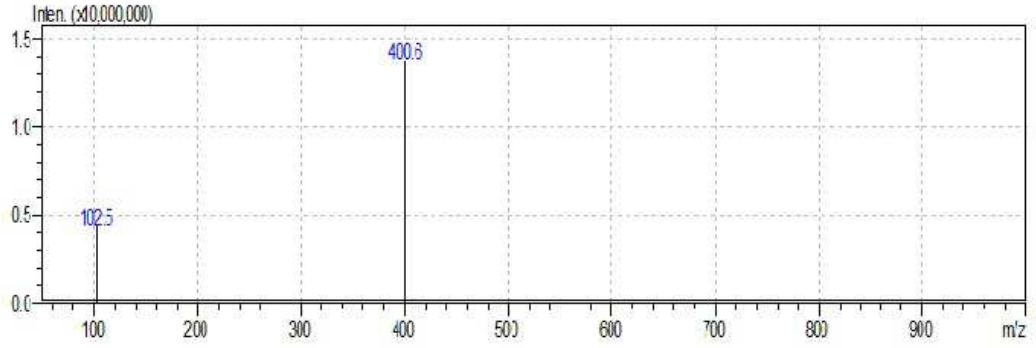

Fig. 4. LC-MS results of degradation products (b) 21-hydroxy deflazacort 400.6 m/z

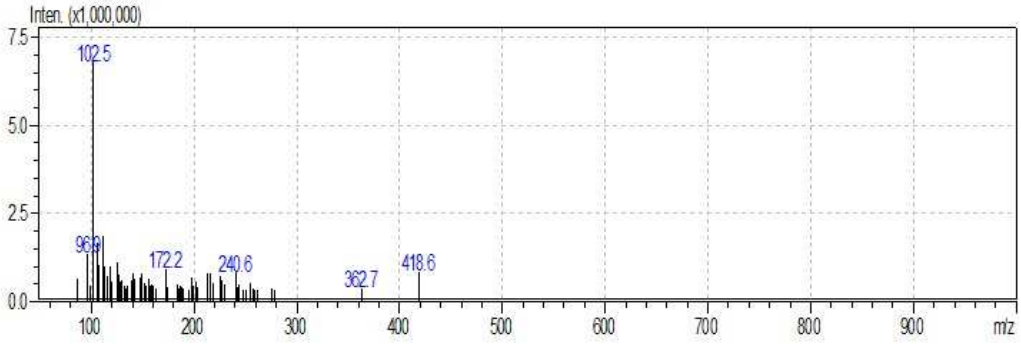

Fig. 4. LC-MS results of degradation products (c) DP2 418 m/z

Method validation:

Solution stability:

Stability of solution was evaluated for the standard solution and test preparation. The solutions were stored at two different conditions: one is ambient and second is at 5^{o} temperatures without protection of light. All samples were tested after 6, 12, 24, and 48 hours. The responses for the aged solution were evaluated by comparison with freshly prepared solution. The assay difference between most aged solution after 48 h and freshly prepared solution was 0.9 % for UPLC. Similarly, solution stability for HPLC was 1.2 %. Absolute percentage assay differences for solution stability study are given in tab. 2.

Tab. 2. Absolute percentage assay difference for solution stability study

Time Interval	Absolute difference in assay for HPLC, (%)		Absolute difference in assay for UPLC, (%)	
	At 5°	At room temperature	At 5°	At room Temperature
After 6 hour	0.65	0.97	0.88	0.92
After 12 hours	0.87	1.27	0.97	1.11
After 24 hours	1.13	1.69	1.09	1.30
After 48 hours	1.33	1.90	1.20	1.70

System suitability:

The suitability of the chromatographic system was tested before each stage of validation. Five replicate of standard preparation were injected and asymmetry, number of theoretical plates and % RSD of peak area were determined. The comparison of HPLC and UPLC results are given in tab. 3.

Tab. 3. Summary of method validation parameter for HPLC and UPLC

	Deflazacort UPLC method			Deflazacort HPLC method		
Method Validation Results (in-house limits)	**% RSD[a] (NMT[b] 2.0)**	**Theoretical Plates (NLT[c] 4200)**	**Peak tailing (NMT[b] 2.0)**	**% RSD[a] (NMT[b] 2.0)**	**Theoretical Plates (NLT[c] 4200)**	**Peak tailing (NMT[b] 2.0)**
Specificity	0.38	6486	1.70	0.26	8428	1.56
Linearity	0.23	6356	1.62	0.48	8256	1.68
LOQ	0.98	6289	1.67	0.68	8369	1.69
Method Precision	0.76	6337	1.63	0.75	8248	1.52
Intermediate Precision	0.68	6249	1.65	0.86	8453	1.63
Accuracy	0.89	6327	1.63	0.85	8385	1.68
Solution Stability	0.44	6298	1.68	0.56	8326	1.69
Robustness	0.63	6325	1.69	0.60	8487	1.58

[a]Relative standard deviation
[b]Not more than
[c]Not less than

Accuracy:

The accuracy of the assay method was evaluated by preparing three different concentration levels corresponding to 50%, 100 %, 150 % (25, 50, 75 µg/mL of deflazacort respectively) of test preparation concentration in triplicate and injecting it in duplicate. The recovery found was between 99-101% and 98-101% for UPLC and HPLC in that order

which is suitable as per ICH guideline Q2 (A). The UPLC and HPLC data for the percentage recovery are shown in the tab. 4.

Tab. 4. Percentage recovery data for UPLC and HPLC accuracy study

Instrument Used	Level %	No	Amount of drug added (µg/ml)	Amount of drug found (µg/ml)	Recovery (%)	Mean Recovery (%)	RSD[a] (%)
UPLC	50	1	25.52	25.45	99.73	99.69	0.057
		2	25.42	25.33	99.65		
	100	1	50.18	50.22	100.08	99.92	0.051
		2	50.26	50.14	99.76		
	150	1	74.89	75.21	100.43	99.83	0.860
		2	76.95	76.35	99.22		
HPLC	50	1	25.02	24.97	99.80	99.86	0.084
		2	25.03	25.01	99.92		
	100	1	50.04	49.76	99.44	99.52	0.113
		2	50.06	49.86	99.60		
	150	1	75.09	74.68	100.03	99.62	0.582
		2	75.05	74.56	99.21		

RSD[a] Relative Standard Deviation

Precision:
The drug product was examined in same day and the results were subjected to statistical analysis to check the repeatability and reproducibility in the means of method precision. The % RSD for deflazacort drug product ware 0.76 and 0.75 using UPLC and HPLC respectively. Intermediate precision was confirmed with inter day and intraday testing of drug tablet. The intra-day precision study was performed in a same day by analyzing three times with six independent assays of test sample against reference material. Inter-day precision of the method was determined by performing the same procedure on three different days.

Linearity:
The linearity of the method was assessed by seven different level concentrations ranging from 20 to 80 µg/mL deflazacort test solutions (40 to 160 % respectively) prepared using stock solution. The slope, Y- intercepts and correlation coefficient were calculated by plotting peak area versus concentration curve. These are Y=18153x + 15478 and Y= 13851x + 530.7 for HPLC and UPLC respectively. The results obtained were used to calculate equation of the regression line by using the linear least squares regression equation.

Limit of detection (LOD) and limit of quantification (LOQ):
LOD and LOQ for deflazacort were determined at signal to noise ratios of 3:1 and 10:1 respectively by injecting series of dilute solutions prepared by serial dilutions of the known concentration. The concentration 0.2 µg/mL and 0.03 µg/mL are LOD level for HPLC and UPLC method respectively. Moreover, 0.16 µg/mL and 1.8 µg/mL are the LOQ level for UPLC and HPLC respectively. A precision evaluation was also carried out at LOQ level by taking six individual preparations and calculating the %RSD of area for deflazacort by both the LC methods.

Robustness:
Robustness study for the developed method was carried out by assaying test solution after slight but deliberate changes in the experimental conditions. The influence of chromatographic parameter (k) was investigated for flow rate, different column lot, amount of methanol, column temperature and amount of water. Changes in system suitability parameters such as theoretical plates, tailing factor and %RSD were evaluated for the method. All the results were found within the acceptance criteria, which suggest that both the methods were highly robust.

CONCLUSION

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for both assay and determination of content uniformity of deflazacort in pharmaceutical formulation. Deflazacort is very much sensitive to pH and degraded immediately after addition of alkaline solution in very mild concentration and comparatively less sensitive to acid solution. Degradation products produced as a result of stress did not interfere with detection of deflazacort and the assay method can thus be regarded as stability indicating. The degradation product 21-hydroxy deflazacort and a new DP-2 degradation product obtained from alkali and acid degradation conditions respectively. These products are confirmed by their molecular ion peaks by LC-ESI-MS. However, chromatographic conditions of both methods are almost same due to method transfer from HPLC to UPLC. Some changes were required to obtain suitability of method by the means of

asymmetry, number of theoretical plates and % RSD. The lower concentration for LOD and LOQ in UPLC method compare to HPLC method shows the greater sensitivity. The total analysis time required by HPLC method is 10 min whereas in UPLC method it reduced to 3 min. which is three fold less than HPLC method. This rapid analytical method establishes the more efficient workflow with the new generation instrument UPLC.

The method was revealed to be selective, precise, sensitive, rapid and linear that was confirmed by the method validation results. The proposed both the chromatographic methods represent good sensitivity, resolution and selectivity in bulk drug as well in pharmaceutical dosage forms. UPLC method is faster and sensitive as compare to HPLC method. The major degradation products observed in acid, alkali and oxidation conditions are eluted at same retention time. The probable structures of both the degradation products are meat with the LC-MS results. The method can be used for the estimation of deflazacort in the form of drug substance as well as drug product.

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