

A Validated Stability-Indicating LC Method for Fluocinonide in the Presence of Degradation Products, Its Process-Related Impurities and Identification of Degradation Products

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Abstract

The objective of the current study was to develop a validated, specific and stability-indicating reverse phase liquid chromatographic method for the quantitative determination of fluocinonide and its related substances. The determination was done for active pharmaceutical ingredient and its pharmaceutical dosage forms in the presence of degradation products, and its process-related impurities. The drug was subjected to stress conditions of hydrolysis (acid and base), oxidation, photolysis and thermal degradation per International Conference on Harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. Significant degradation was observed during acid, base hydrolysis, and peroxide degradation. The major degradants were identified by LC-MS, FTIR and ¹H/¹³C NMR spectral analysis. The chromatographic conditions were optimized using an impurity-spiked solution and the samples generated from forced degradation studies. In the developed HPLC method, the resolution between fluocinonide and its process-related impurities, (namely imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8) and its degradation products was found to be greater than 2.0. The chromatographic separation was achieved on a C18, 250 mm × 4.6 mm, 5 μm column. The LC method employed a linear gradient elution and the detection wavelength was set at 240 nm. The stress samples were assayed against a qualified reference standard and the mass balance was found to be close to 99.3%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

Keywords: Fluocinonide, RP-LC, LC-MS, Forced Degradation, Validation, Stability-Indicating

1. Introduction

Fluocinonide, pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6, 9-difluoro-11-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6α, 11β, 16α)-, is a potent glucocorticoid steroid used topically as anti-inflammatory agent for the treatment of skin disorders such as eczema. It relieves itching, dryness, redness, crusting, scaling, inflammation and discomfort. Fluocinonide is a white or almost white crystalline powder. It is practically insoluble in water; slightly soluble in absolute ethanol. There are seven classes of topical corticosteroids ranked according to their potency based vasoconstrictor assays. Fluocinonide is a fluorinated, highly potent, corticosteroid. It ranks as

a “high-potency” (second-highest rank) topical corticosteroid [1]. Corticosteroids have multiple actions, including anti-inflammatory and anti-proliferative effects. When applied topically for at least one week, fluocinonide is effective in the treatment of inflammation and itching caused by a number of skin conditions such as allergic reactions, eczema and psoriasis. Minimal amounts should be used for a minimal length of time to avoid the occurrence of adverse effects. Fluocinonide is also used in veterinary medicine. It is used for treatment of allergies in dogs. Natural systemic cortisol concentrations can be suppressed for weeks after one week of topical exposure.

A chromatographic assay method has appeared in US

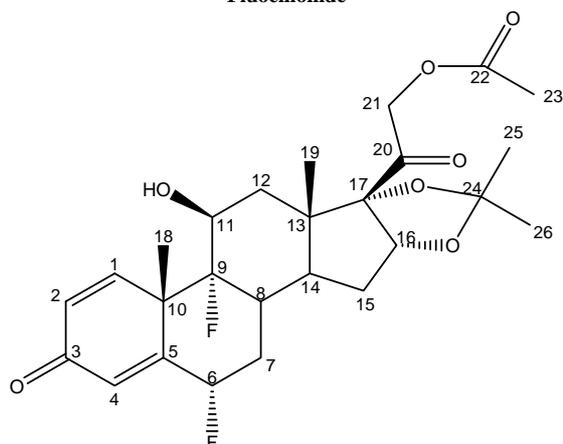
pharmacopeia for the quantification of fluocinonide in drug substance and its pharmaceutical topical dosage forms [2]. Few analytical methods were available in literature for the quantification of fluocinonide and fluclo-cortisone acetate. Shek E. *et al.* has described an HPLC procedure to chromatograph fluocinonide as well as triamcinolone acetonide and diflorasone diacetate [3-6]. In addition, Shek E. *et al.* reported the stability indicating LC method for triple corticoid integrated system in a cream. [3] in which Shek. E. *et al.* described the quantification of fluocinonide, procinonide and ciprocinonide and expected the major degradation product under hydrolytic conditions would be fluocinolone acetonide. Further study was not carried out to confirm this impurity. The present study was carried out to describe detailed degradation studies as per ICH guidelines and developed a suitable LC method for the separation and estimation of both process related impurities and degradation impurities. Interestingly author observed two major degradation products in acid, base hydrolysis and peroxide degradation of fluocinonide. The major degradation products of fluocinonide were isolated using preparative HPLC and structure elucidation was performed for the same using advanced spectral techniques such as NMR, LC-MS and IR. The purpose of the present research work was to develop a suitable single stability-indicating LC method for the determination of fluocinonide and its related substances and structure elucidation of major degradation products. The developed LC method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Forced degradation studies were performed on the drug substance and drug products to show the stability-indicating nature of the method [7-13]. These studies were performed in accordance with established ICH guidelines [14-15].

2. Experimental

2.1. Chemicals

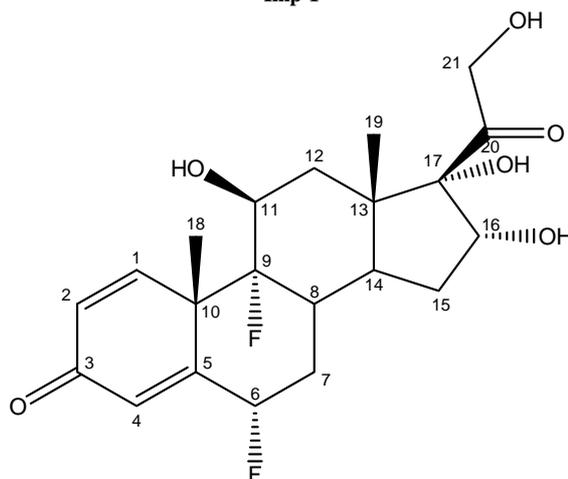
Samples of fluocinonide and its related impurities were received from Versapharm Incorporated, Warminster, PA, USA. (**Figure 1**). All of the impurities and the fluocinonide standard were of > 90% purity and are as follows:-, fluocinonide (99.6%), imp-1 (95.9%), imp-2 (99.3%), imp-3 (93.7%), imp-4 (99.5%) imp-5 (96.7%), imp-6 (98.5%), imp-7 (97.8%) and imp-8 (99.2%). Commercially available fluocinonide ointment and fluocinonide solution (LIDEX) were purchased for this study. In addition, HPLC grade acetonitrile and methanol were purchased from Merck, (Darmstadt, Germany). Analytical reagent grade sodium dihydrogen phosphate monohydrate, phosphoric acid and acetic acid were purchased

Fluocinonide



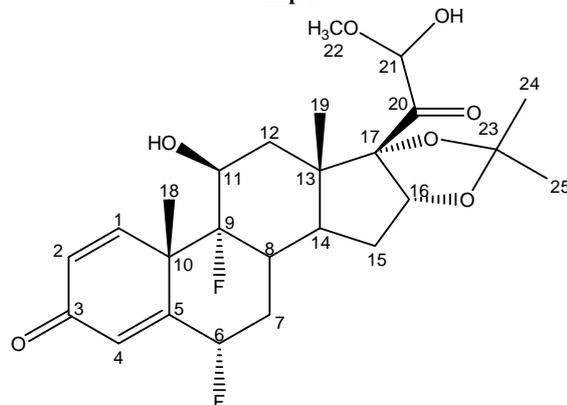
(a) pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6, 9-difluoro-11-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)], (6 α , 11 β , 16 α)-. Molecular weight: 494.52.

Imp-1

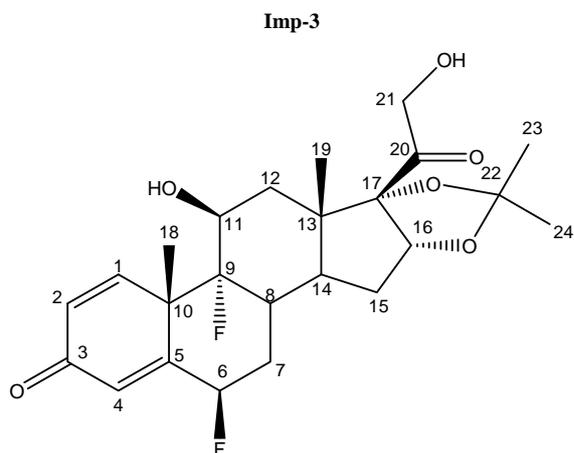


(b) pregna-1, 4-diene-3, 20-dione, -6, 9-difluoro-11, 16, 17, 21-tetrahydroxy-, (6 α , 11 β , 16 α)-. Molecular weight: 412.42.

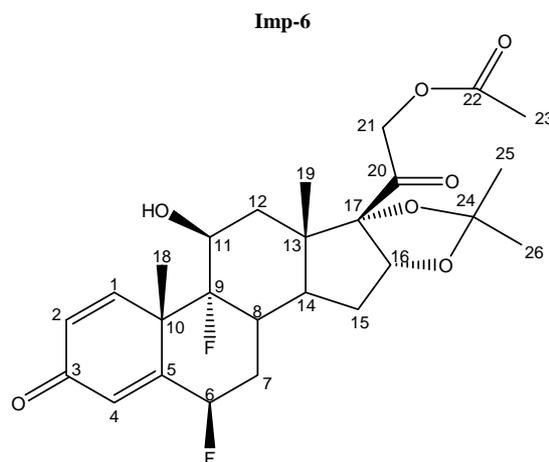
Imp-2



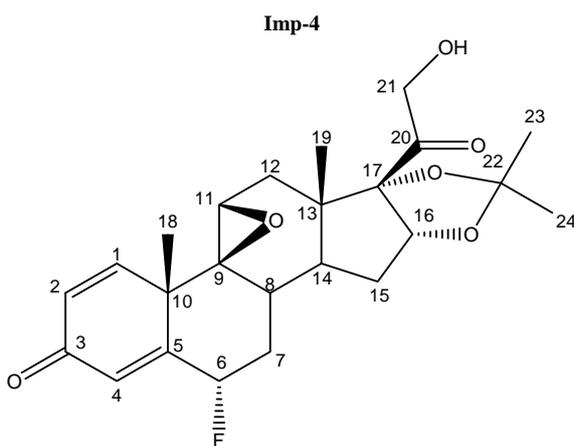
(c) pregna-1, 4-diene-3, 20-dione, -6, 9-difluoro-11, 21-dihydroxy-21methoxy-16, 17-[(1-methylethylidene)bis(oxy)]-(6 α , 11 β , 16 α) Molecular weight: 482.51.



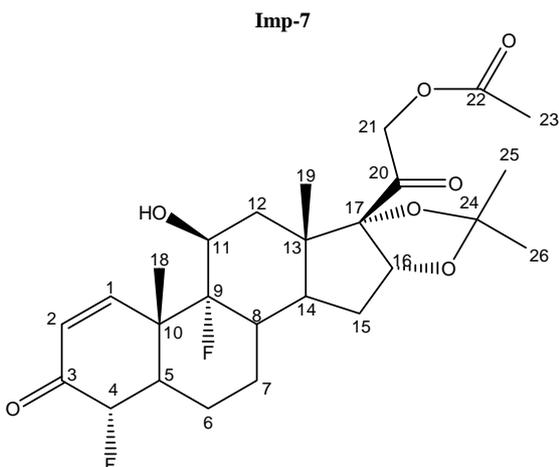
(d) pregna-1, 4-diene-3, 20-dione, -6, 9-difluoro-11, 21-dihydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6 β , 11 β , 16 α)-. Molecular weight: 452.49.



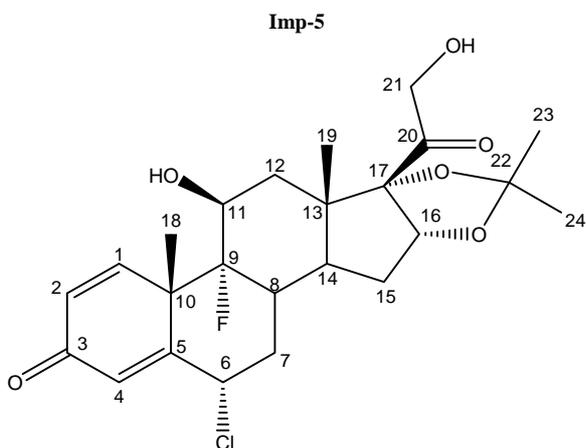
(g) pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6, 9-difluoro-11-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6 β , 11 β , 16 α)-. Molecular weight: 494.52.



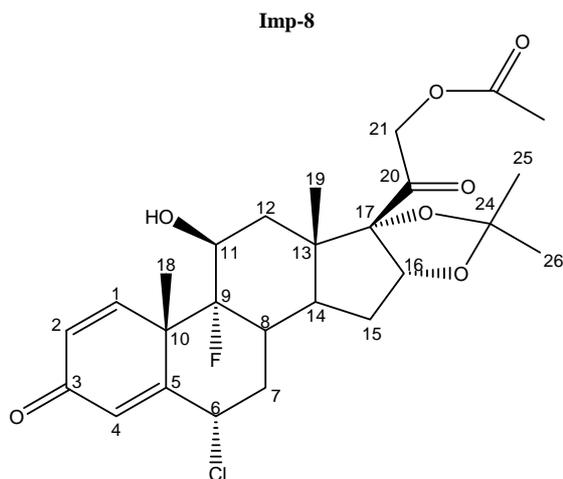
(e) pregna-1, 4-diene-3, 20-dione, -6-fluoro-9, 11 epoxy, -21-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6 α , 11 β , 16 α)-. Molecular weight: 432.48.



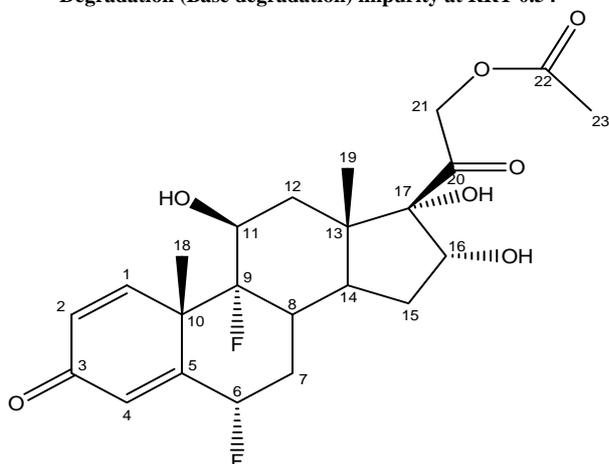
(h) pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-4, 9-difluoro-11-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (4 α , 11 β , 16 α)-. Molecular weight: 496.54.



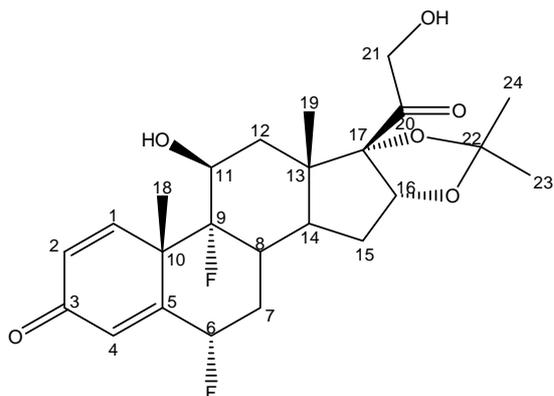
(f) pregna-1, 4-diene-3, 20-dione, -6 chloro, 9-fluoro-11, 21-dihydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6 α , 11 β , 16 α)-. Molecular weight: 468.94.



(i) pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6-Chloro, 9-fluoro-11-hydroxy-16, 17-[(1-methylethylidene)bis(oxy)]-, (6 α , 11 β , 16 α)-. Molecular weight: 510.18.

Degradation (Base degradation) impurity at RRT 0.54

(j) Pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6, 9-difluoro-11, 16, 17-trihydroxy-(6 α , 11 β , 16 α)- Molecular weight: 454.46.

Degradation (Acid and peroxide degradation) impurity at RRT 0.60

(k) Pregna-1, 4-diene-3, 20-dione, 6, 9-difluoro-11, 21 dihydroxy-16, 17-[(1-Methylethylidene) bis (oxy)]-, (6 α , 11 β , 16 α)- Molecular weight: 452.49.

Figure 1. Structures and names of fluocinonide and its impurities.

from Merck. Highly pure water was prepared with the Millipore Milli-Q Plus water purification system.

2.2. Equipment

The LC system used for method development, forced degradation studies and method validation consisted of a Waters 2695 binary pump with an auto sampler and a 2996 photo diode array detector (PDA). The output signal was monitored and processed using Empower software on a Pentium computer (Digital equipment Co.). Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were carried out in a dry air oven (Lindberg-Blue, USA).

2.3. Chromatographic Conditions

A Waters symmetry C18 250 mm \times 4.6 mm, 5 μ m column was used with a mobile phase containing a gradient of solvents A and B. Solvent A was composed of water, with its pH adjusted to 3.0 with orthophosphoric acid. Water and acetonitrile in the ratio of 150:850 (v/v) was used as solvent B. The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/40, 30/80, 35/80, 35.1/40 and 45/40 (time (min)/%B). The column temperature was maintained at 27°C and the detection wavelength was set at 240 nm. The injection volume was 20 μ l. The diluent consisted of water and acetonitrile in a ratio of 50:50 (v/v).

2.4. LC-MS Conditions

The LC-MS system (Agilent 1100 series liquid chromatography system coupled with a 6400 series triple quadrupole mass spectrometer) was used for the identification of unknown compounds formed during forced degradation. A Waters symmetry C18 250 mm \times 4.6 mm, 5 μ m column was used as the stationary phase. Water, acetonitrile and acetic acid in a ratio of 60:40:1 (v/v/v) was used as the mobile phase. A mixture of water and acetonitrile in a 50:50 (v/v) ratio was used as the diluent. The flow rate was 1.0 ml/min. The analysis was performed in positive and negative electrospray ionization modes. The capillary and cone voltages were 3.5 kV and 25 V, respectively. The source and dissolution temperatures were 120°C and 350°C, respectively and the dissolution gas flow was 500 l h⁻¹.

2.5. Preparation of Standard Solutions and Sample Solutions

A stock solution of fluocinonide (2.5 mg/ml) was prepared by dissolving the appropriate amount of fluocinonide solid in the diluent. Working solutions of 250 and 25 μ g/ml were prepared from the stock solution for the determinations of related substances and assay respectively. A stock solution of impurity (mixture of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8) at 0.25 mg/ml was also prepared in the diluent.

Fluocinonide solution sample preparation: The solution (2.5 g) equivalent to 2.5 mg of drug was transferred into a 10 ml volumetric flask, and 2 ml of diluent was added. The flask was attached to a rotary shaker and shaken for 10 min to mix completely. The mixture was sonicated for 2 min and then diluted to the appropriate volume with diluent to make a solution containing 0.25 mg/ml. The solution was filtered through a 0.45 μ Nylon 66 membrane filter.

Fluocinonide ointment sample preparation: Sample (2.5 g ointment) equivalent to 2.5 mg of drug was transferred into a 50 ml screw test tube, and 5 ml of diluent was added. The test tube was heated for 5 min at 65°C, cooled to room temperature, cyclomix for 2 min and repeated the procedure once again, cool to room temp and added 5ml of diluent cyclomix for 3 min. The solution was filtered through a 0.45 μ Nylon 66 membrane filter. These solutions were used for the related substances estimation of fluocinonide. For assay analysis 19-norethindrone was used as internal standard.

2.6. Stress Studies/Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [14]. The specificity of the developed LC method for fluocinonide was determined in the presence of its impurities (namely imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8) and degradation products. Forced degradation studies were also performed on fluocinonide to provide an indication of the stability- indicating property and specificity of the proposed method [7-12]. The stress conditions employed for the degradation study included light (carried out as per ICH Q1B), heat (60°C), acid hydrolysis (1M HCl), base hydrolysis (0.1M NaOH) and oxidation (5% H₂O₂). For heat and light studies, the samples were exposed for 10 days, whereas the samples were treated for 48 h for acid hydrolysis and for oxidation, 30 minutes for base hydrolysis. The peak purity of the fluocinonide stressed samples was checked by using a Waters 2996 photo diode array detector (PDA). The purity angle was within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the analyte peak.

Assays were carried out for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) was calculated for all of the samples.

2.7. Method Validation

The proposed method was validated per ICH guide lines [14-15].

2.7.1. Precision

The precision of the related substance method was investigated by injecting six individual preparations of (250 μ g/ml) fluocinonide spiked with 0.15% each of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8. The %RSD of the areas of each imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 was calculated.

The intermediate precision of the method was evalu-

ated using a different analyst and instrument located within the same laboratory.

The precision of the assay method was evaluated by carrying out six independent assays of a test sample of fluocinonide against a qualified reference standard. The %RSD of six obtained assay values was calculated.

2.7.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study was also carried at the LOQ level by injecting six individual preparations of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 and calculating the %RSD of the areas.

2.7.3. Linearity

Linearity test solutions for the assay method were prepared from a stock solution at five concentration levels from 50 to 150% of the assay analyte concentration (12.5, 20, 25, 30, and 37.5 μ g/ml). The peak area versus concentration data was analyzed with least-squares linear regression.

Linearity test solutions for the related substance method were prepared by diluting the impurity stock solution (2.5) to the required concentrations. The solutions were prepared at eight concentration levels from the LOQ to 150% of the specification level (LOQ, 0.05%, 0.10%, 0.15%, 0.20%, 0.25% and 0.3%). The slope and y-intercept of the calibration curve are reported.

2.7.4. Accuracy

The accuracy of the assay method was evaluated in triplicate at four concentration levels (LOQ, 12.5, 25 and 37.5 μ g/ml), and the percentage recoveries were calculated.

The drug substance did not show the presence of imp-1, imp-2, imp-3, imp-4, imp-5, imp-7 and imp-8, but contained 0.29% of imp-6. Standard addition and recovery experiments were conducted to determine the accuracy of the related substance method for the quantification of all eight impurities (imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8) in the drug substance as well as in the drug product. The study was carried out in triplicate at 0.075%, 0.15% and 0.225% of the analyte concentration (250 μ g/ml). The percentage of recoveries for imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 were calculated.

2.7.5. Robustness

To determine the robustness of the developed method,

the experimental conditions were altered and the resolution between fluocinonide and imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 was evaluated. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.1 units (0.9 and 1.1 ml/min). The effect of pH on the resolution of the impurities was studied by varying the pH by ± 0.1 units (buffer pH of 2.9 and 3.1). The effect of the column temperature on the resolution was studied at 22°C and 32°C instead of 27°C. In all these varied conditions, the components of the mobile phase remained constant, as outlined in Subsection 2.3.

2.7.6. Solution Stability and Mobile Phase Stability

The solution stability of fluocinonide in the assay method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48h. The same sample solutions were assayed for in 6h intervals over the study period. The mobile phase stability was also examined by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6h intervals up to 48h. The prepared mobile phase remained constant during the study period. The %RSD of the fluocinonide assay was calculated for the mobile phase and solution stability experiments.

The solution stability of fluocinonide and its impurities in the related substance method was carried out by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature for 48h. The con-

tent of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 was determined at 6h intervals up to the study period.

The mobile phase stability was also investigated for 48h by injecting the freshly prepared sample solutions for every 6h interval. The content of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 was determined in the test solutions. The prepared mobile phase remained constant during the study period.

3. Results and Discussion

3.1. Method Development and Optimization

All the impurities and fluocinonide solutions were prepared in diluent at a concentration of 10 ppm and scanned in UV-visible spectrophotometer; all the eight impurities and fluocinonide had a UV maxima at around 240 nm (**Figure 2**). Hence detection at 240 nm was selected for method development purpose.

The main objective of the chromatographic method was to separate imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7, imp-8 and the generated degradation products from the analyte peak during stress studies. Impurities and degradation products were co-eluted by using different stationary phases, such as C8, cyano and phenyl with various mobile phases with buffers, such as phosphate and acetate with different pH values (3-7), and organic modifiers, including acetonitrile and methanol.

United States Pharmacopeia stated LC method for the

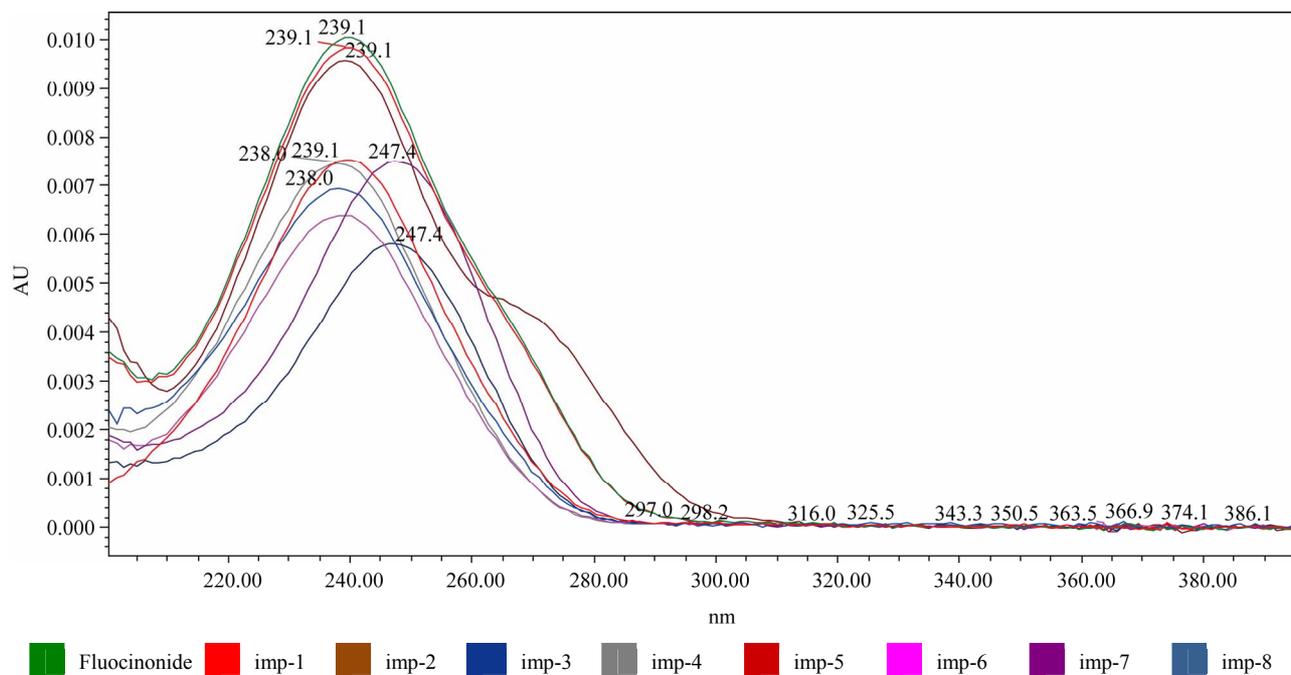
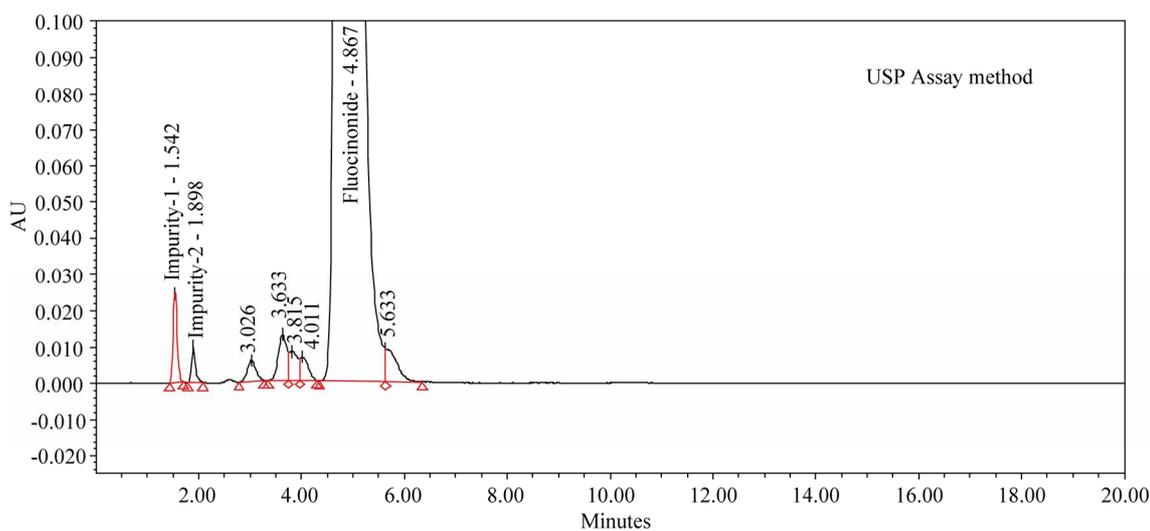


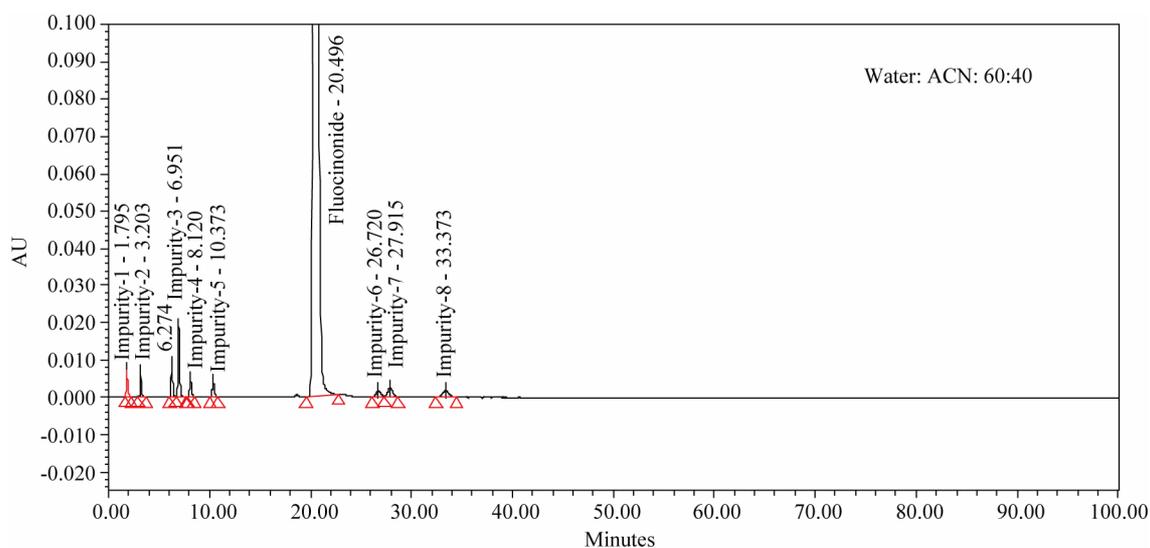
Figure 2. Typical spectra of fluocinonide and its impurities.

estimation of related substances of fluocinonide for drug substance [2]. Hence this was chosen as initial experiment. The method consists of water and acetonitrile (50:50, v/v) as mobile phase at a flow rate of 2.0 ml/min with Waters bondapak 300 mm \times 3.9 mm ID column and 10 μ m particle size C18 stationary phase. When an impurity spiked solution of fluocinonide was injected. Imp-1 and Imp-2 were eluted at void with retention times of 0.8 and 1.3, imp-6 and imp-7 were co eluted (**Figure 3**). In acid degradation sample the resolution between one of the major degradant and imp-3 was very less ($R_s < 1.2$). To improve the resolution between impurities and retention of imp-1 and imp-2, particle size of the column was decreased to 5 μ m and flow rate was decreased to 1.0 ml/min and injected the impurity spiked solution. The resolution between Imp-6 and imp-7 was slightly

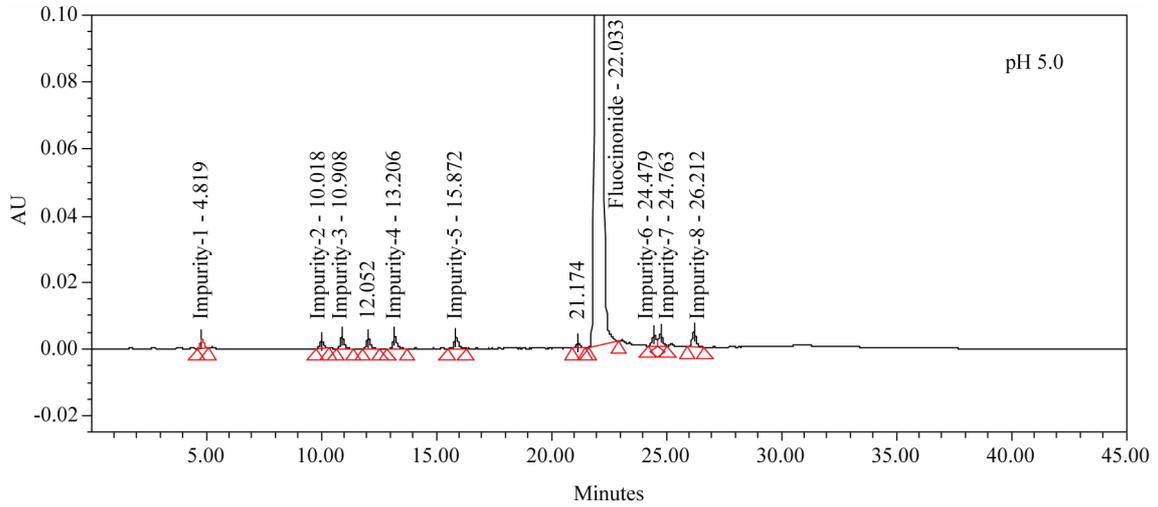
improved but there is no improvement in the retention of imp-1 and imp-2. To improve the retention times of imp-1 and imp-2 acetonitrile content in mobile phase was decreased to 30% (water: acetonitrile: 70:30) and injected impurity spiked solution. The retention of imp-1 and imp-2 was improved but fluocinonide peak was eluted at round 60 min. Isocratic trails were not successful in achieving a favorable resolution between the impurities and retention of imp-1, imp-2 and fluocinonide. Therefore, a gradient method was selected using buffer (0.01M ammonium acetate, pH 7.0) as mobile phase A and water and acetonitrile in a ratio of 100:900, v/v as mobile phase B, retention time of imp-1 and imp-2 were improved but imp-6 and imp-7 were almost co eluted. To improve the resolution between Imp-6 and Imp-7 buffer pH adjusted to acidic side (0.01M NaH_2PO_4 , pH 5.0),



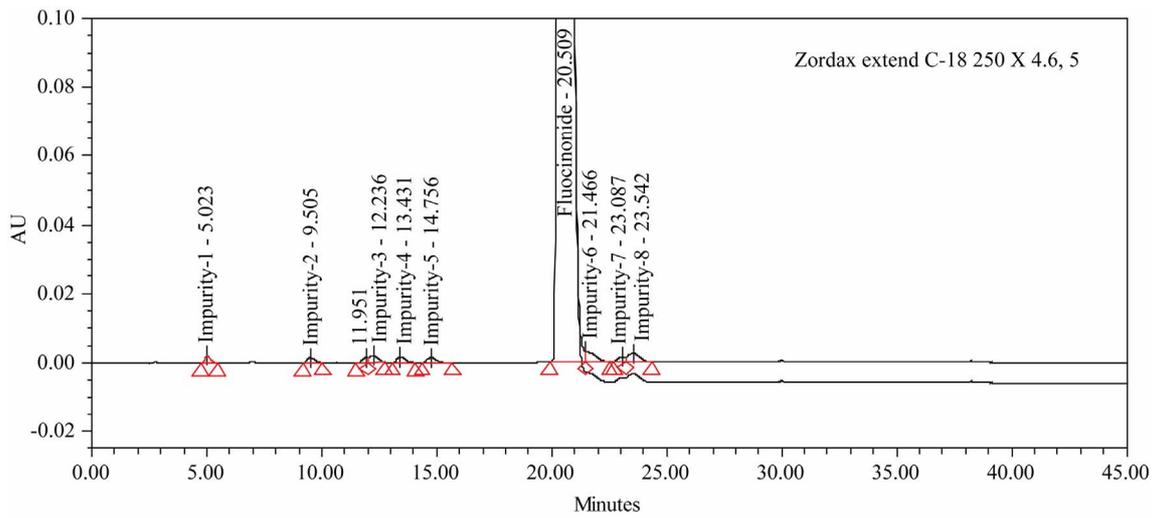
(a)



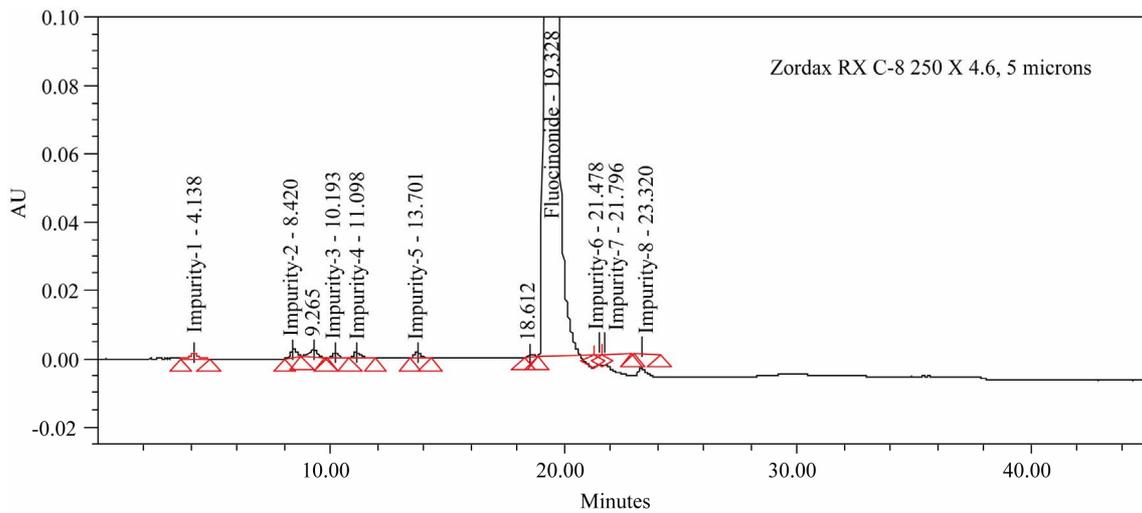
(b)



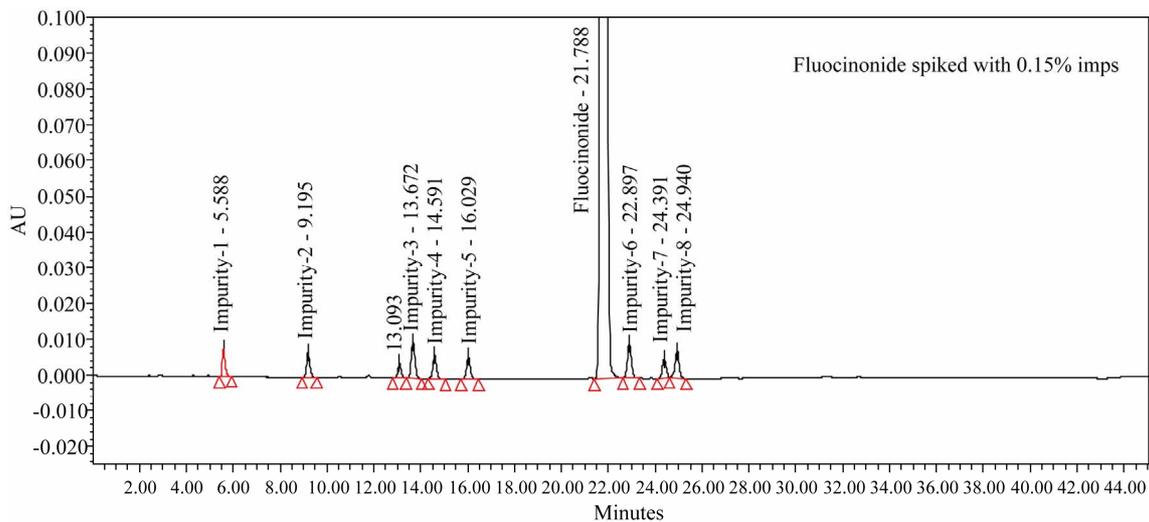
(c)



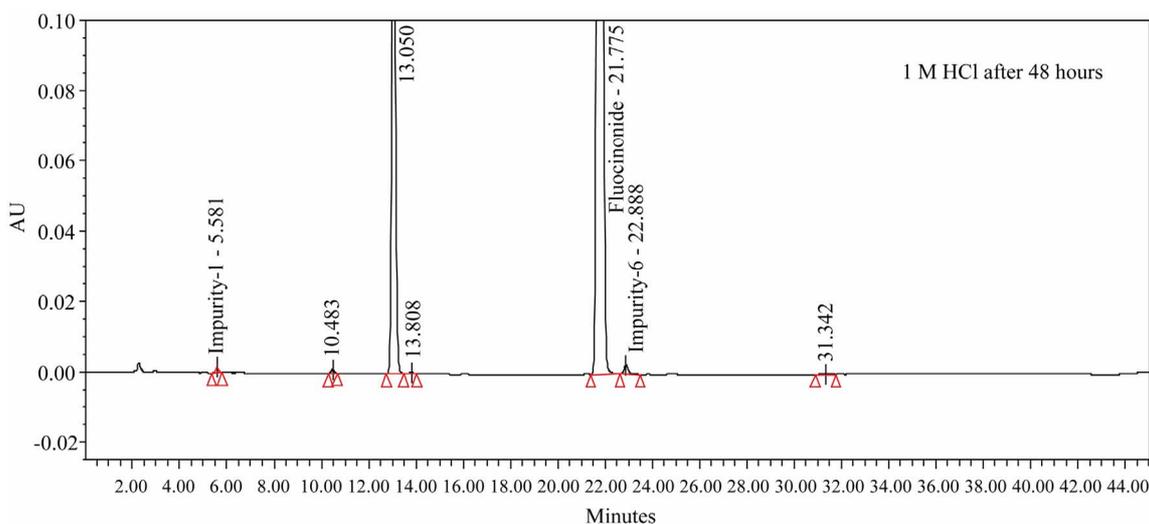
(d)



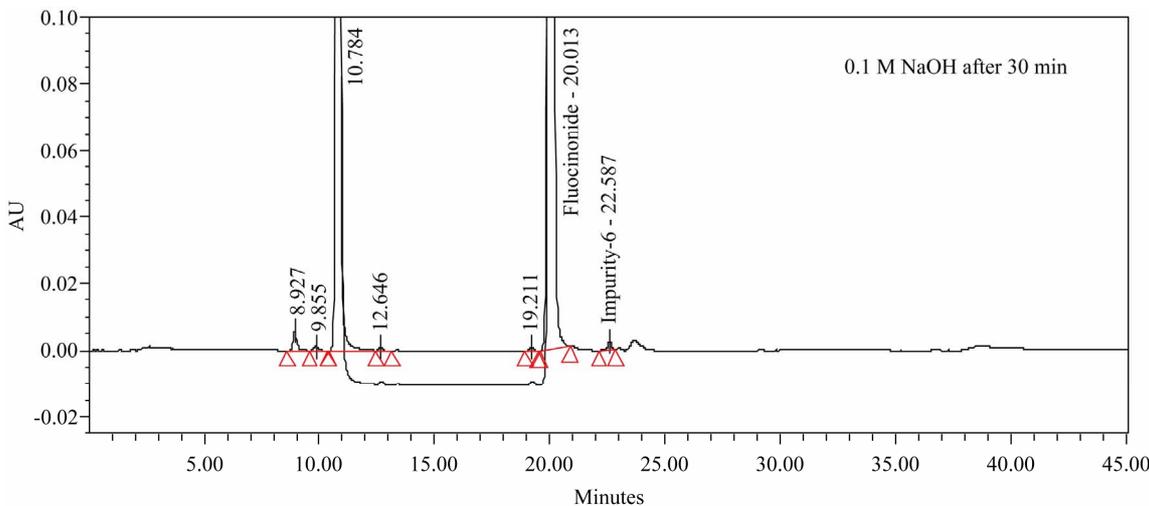
(e)



(f)



(g)



(h)

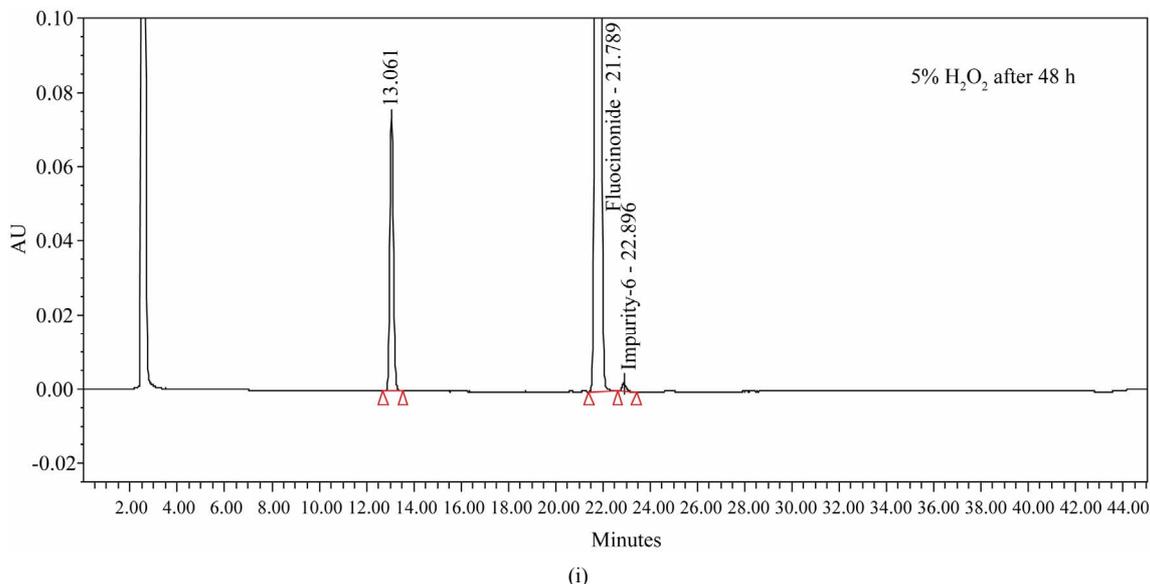


Figure 3. (a-e) Typical chromatograms from the method development trials; (f-i) Typical chromatograms of system suitability and stressed fluocinonide samples.

slight improvement in resolution between imp-6 and imp-7 was observed ($R_s < 1.0$). To further improve the resolution buffer pH was adjusted to 3.0 (0.01% phosphoric acid, pH 3.0). The resolution observed was 1.8. Therefore, a pH value of 3.0 was selected for further method development. Different gradient programs were investigated and satisfactory results were obtained when a gradient program of 0/40, 30/80, 35/80, 35.1/40 and 45/40 (time (min)/%B) was used.

Under the above described conditions, impurity spiked solution and degradation samples were injected on a cyano, phenyl, C8 and C18 columns having different carbon loadings. On the cyano and phenyl columns, imp-6 and imp-7 were co-eluted however, on C8 column (Zorbax RX C8 with carbon loading ~8%), and C18 column (Agilent extend C18 column (carbon loading ~12%) the resolution between imp-6 and imp-7 was poor ($R_s < 1.2$). In contrast, when a Waters symmetry C18 250 mm x 4.6 mm, 5 μ m column (carbon loading ~18%) was used, satisfactory results were obtained. Based on these experiments, the conditions were further optimized as described below.

Waters symmetry C18 250 mm x 4.6 mm, 5 μ m column was used as the stationary phase. The Mobile phase A consisted of water, and its pH was adjusted to 3.0 using phosphoric acid. The mobile phase B contained a mixture of water and acetonitrile in the ratio of 150:850 (v/v). The flow rate of the mobile phase was 1.0 ml/min with a gradient program of 0/40, 30/80, 35/80, 35.1/40 and 45/40 (time (min)/%B). The column temperature was maintained at 27°C and the detection was monitored at a wavelength of 240 nm. The injection volume was 20 μ l.

Using the optimized conditions, fluocinonide, imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 were well separated with a resolution of greater than 2 and typical retention times for imp-1, imp-2, imp-3, imp-4, imp-5, fluocinonide, imp-6, imp-7 and imp-8, of about 5.5, 9.2, 13.7, 14.6, 16.0, 21.8, 22.9, 24.4 and 24.9 min, respectively. The system suitability results are given in **Table 1** and the developed LC method was determined to be specific for fluocinonide and the eight impurities, imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8. (**Table 2**)

Table 1. System suitability report.

Compound	USP Resolution (R_s)	USP Tailing factor	No. of theoretical plates (USP tangent method)
Imp-1	-	1.2	11248
Imp-2	14.9	1.2	20064
Imp-3	16.2	1.1	37955
Imp-4	3.2	1.1	44306
Imp-5	5.0	1.1	52387
Fluocinonide	19.5	1.1	83484
Imp-6	3.6	1.1	87925
Imp-7	4.8	1.0	101784
Imp-8	2.1	1.0	99607

Table 2. Summary of forced degradation results.

Stress condition	Time	% Assay of active substance	Mass balance (%assay + %impurities + % degradation products)	Remarks
Acid hydrolysis (1 M HCl)	48 h	86.5	99.4	One major degradation product was formed.
Base hydrolysis (0.1 M NaOH)	30 min	74.7	99.3	One major degradation product was formed.
Oxidation (5% H ₂ O ₂)	48 h	90.8	99.3	One major degradation product was formed.
Thermal (60°C)	10 days	99.3	99.8	No degradation products formed
Light (photolytic degradation)	10 days	99.2	99.7	No degradation products formed

3.2. Method Validation

3.2.1. Precision

The %RSD of fluocinonide during the assay method precision study was within 0.5% and the %RSD values of the area of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 in the related substance method precision study were within 1.0%. The %RSD of the results obtained in the intermediate precision study was within 0.8% and the %RSD of the areas of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 were well within 1.8%, revealing the high precision of the method.

3.2.2. Limit of Detection and Limit of Quantification

The limits of detection and quantification of fluocinonide, imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 (analyte concentration of 250 µg/ml) for a 20 µl injection volume are given in **Table 3**. The precision at the LOQ concentration for imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 was below 2.2%.

3.2.3. Linearity

The linear calibration plot for the assay method was obtained over the tested calibration range (12.5-37.5 µg/ml) and the obtained correlation coefficient was greater than 0.999. The results revealed an excellent correlation between the peak area and analyte concentration. The slope and y-intercept of the calibration curve for the ratio of fluocinonide area and internal standard area were 0.0392 and 0.0286 respectively.

The linear calibration plot for the related substance method was determined over the calibration ranges (LOQ to 0.3%) for imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8, a correlation coefficient of greater than 0.99 was obtained. The linearity was checked for the related substance method over the same concentration range for three consecutive days. The %RSD values of the slope and y-intercept of the calibration curves were 2.5 and 4, respectively. These results showed

an excellent correlation between the peak areas and concentrations of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8.

3.2.4. Accuracy

The percentage recovery of fluocinonide in the drug substance and product ranged from 98.5 to 101.6 and from 96.2 to 101.6, respectively. The percentage recoveries of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 in the drug substance and product ranged from 95.9 to 102.1 and from 96.3 to 102.5 respectively. The HPLC chromatograms of spiked samples at the 0.15% level of all four impurities in the fluocinonide drug substance sample are shown in **Figure 3**.

3.2.5. Robustness

In all of the deliberately varied chromatographic conditions carried out as described in Subsection 2.3 (flow rate, pH and column temperature), the resolution between the any two peaks in system suitability solution was greater than 2.0, illustrating the robustness of the method. The assay variability of fluocinonide and the impurities was within ± 1% and within ± 2%, respectively.

3.2.6. Solution Stability and Mobile Phase Stability

The %RSD of assaying fluocinonide during the solution stability and mobile phase stability experiments was within 1%. No significant changes were observed in the content of Imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7 and imp-8 during the solution stability and mobile phase stability experiments when performed using the related substances method. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays and related substance determinations were stable up to 48 h.

3.2.7. Results of Forced Degradation Studies

Degradation was not observed in fluocinonide stressed

Table 3. 1H-NMR and 13C-NMR assignments for fluocinonide and its degradation impurities.

Position	Impurity at RRT 0.54 (Base Deg imp)		Impurity at RRT 0.60 (Acid Deg imp)		Fluocinonide	
	δ C	δ H(J/HZ)	δ C	δ H(J/HZ)	δ C	δ H(J/HZ)
1	154.5	7.1(1H,d, 10)	155.4	7.15(1H,d,10)	155.9	7.2(1H,d,10)
2	128.8	6.3(1H,dd,10,1.5)	128.4	6.4(1H,dd,10,1.4)	127.8	6.4(1H,dd,10,1.4)
3	184.6	-	185.5	-	184.1	-
4	123.5	6.15(1H,s)	124.1	6.5(1H,s)	124.9	6.5(1H,s)
5	163.4	-	163.8	-	163.1	-
6	87.1	5.4(1H,dd)	87.5	5.4(1H,dd)	87.8	5.4(1H,dd)
7	34.2	1.58(2H,m)	33.7	1.58(2H,m)	34.4	1.58(2H,m)
8	31.5	1.68(1H,m)	32.3	1.68(1H,m)	32.3	1.67(1H,m)
9	101.5	-	100.7	-	100.9	-
10	47.4	-	47.9	-	47.9	-
11	70.1	4.45(1H,m)	70.9	4.45(1H,m)	70.1	4.45(1H,m)
12	33.9	1.63(2H,m)	33.5	1.63(2H,m)	33.6	1.63(2H,m)
13	36.1	-	36.6	-	36.8	-
14	36.5	1.4(1H,m)	37.2	1.4(1H,m)	37.7	1.4(1H,m)
15	26.9	1.75(2H,m)	26	1.75(2H,m)	27	1.75(2H,m)
16	74.1	4.76(1H,m)	81.5	4.91(1H,m)	80.5	4.90(1H,m)
17	103.1	-	96.8	-	96.5	-
18	18.1	0.9(3H,s)	18.5	0.9(3H,s)	18.4	0.9(3H,s)
19	15.6	1.5(3H,s)	14.2	1.5(3H,s)	14.1	1.5(3H,s)
20	212.9	-	211.5	-	211.8	-
21	65.4	4.95(2H,s)	64.1	4.20,4.70(dd, 2H,19,6)	64.8	4.85(2H,s)
22	170.5	-	108.6	-	171.2	-
23	20.8	2.2(3H,s)	26.6	1.25(3H,s)	26.6	2.1(3H,s)
24	-	-	26.6	1.45(3H,s)	170.1	-
25	-	-	-	-	26.2	1.20(3H,s)
26	-	-	-	-	26.2	1.42(3H,s)
11-OH	-	5.27(H,bd)	-	5.29(1H,bd)	-	5.25(1H,bd)
16-OH	-	4.65(1H,d,6)	-	-	-	-
17-OH	-	4.85(1H,s)	-	-	-	-
21-OH	-	-	-	4.95(1H,t,6)	-	-

samples subjected to light and heat. Significant degradation of the drug substance and product was detected under acid, base hydrolysis and peroxide degradation, leading to the formation of two major unknown degradation products one is at 0.54 RRT (due to base hydrolysis) and another one is at 0.60 RRT (due to acid hydrolysis and peroxide degradation) (**Figure 1**). Fluocinonide is very sensitive towards base compared to acid and peroxide, within 30 minutes fluocinonide degraded 25% to an unknown impurity at RRT 0.54. Peak purity test results derived from the PDA detector, confirmed that the fluocinonide peak and the degraded peaks were homogeneous and pure in all of the analyzed stress samples. Assay studies were carried out for the stress samples against a qualified reference standard of fluocinonide.

The mass balance of the stressed samples was close to 99.3%. The assay of fluocinonide was unaffected in the presence of imp-1, imp-2, imp-3, imp-4, imp-5, imp-6, imp-7, imp-8 and its degradation products, confirming the stability- indicating power of the developed method.

3.2.8. Identification of Major Degradation Products Formed in Acid Hydrolysis, Peroxide Degradation (RRT ~0.60) and Base Hydrolysis (RRT ~0.54).

Degradation product in acid hydrolysis (RRT ~0.60) and base hydrolysis (RRT ~0.54) were isolated using a Shimadzu LC-8A preparative liquid chromatograph equipped with SPD-10A VP, PDA detector (Shimadzu corporation, Japan), Symmetry C18 (250 mm long × 19 mm i.d) preparative column packed with 7 micron particle size (Waters, USA). A mixture of water and acetonitrile in a 50:50 (v/v) ratio was used as the mobile phase. The flow rate was 10 ml/min. The detection was carried out at 240 nm, Injection volume is 10 ml. Peak cut criteria was set based on peak retention time. Fractions > 95% purity were pooled together and concentrated by rotavapour to remove solvents. Concentrated fraction was passed through the preparative HPLC column again, the elute was concentrated using rotavapour to remove solvents then lyophilized using freeze dryer to obtain a white powder with > 98% purity.

3.2.9. Structure Elucidation of Degradation Impurity at RRT ~0.54

A LCMS study was carried to determine the m/z value of the major degradation product formed under base hydrolysis using an Agilent 1100 series liquid chromatography system coupled with a 6400 series triple quadrupole mass spectrometer. The volatile mobile phase contained water, acetonitrile and acetic acid in a ratio of 60:40:1 (v/v/v) and the conditions were described in section 2.4. The molecular ion peak m/z value obtained for

the degradation product resolving at 0.54 RRT is 455 $[(MH)]^+$, in ESI positive mode corresponding to a molecular weights of 454.

In 1H -NMR and ^{13}C -NMR spectra of new impurity shows chemical shift similarity with fluocinonide, except signals corresponding to C-16 and C-17. In ^{13}C -NMR C-16 and C-17 carbons resonated at δ 74.1 ppm and δ 103.1 ppm respectively (where as C-16 and C-17 carbons in fluocinonide at δ 80.5 ppm and 96.5 ppm). In addition, the hydroxyl groups on C-16 and C-17 resonated as doublet at 4.65 ppm and as a singlet at 4.85 ppm respectively. Comparative 1H -NMR and ^{13}C -NMR spectral data for fluocinonide and RRT ~0.54 is given in **Table 3**.

The IR Spectra performed on dispersion KBr of RRT ~0.54 degradant show the following absorption bands 3415, 3398 ~ (broad, OH Stretching) when compared to fluocinonide IR spectrum, a broad signal with a small splitting (doublet) was observed in the region of 3200-3500 which is a clear indication of 2 additional -OH groups, 2950-2850 (CH, CH₂ and CH₃ Stretching) 1720 (C = O Saturated keto group), 1660 (C = O Conjugation) 1630 and 1610 (C = C Stretching)

The elemental analysis data calculated for C₂₃H₂₈F₂O₇ (454.46): C, 60.79; H, 6.21; F, 8.36; O, 24.64 and found: C, 60.74; H, 6.18. From the above spectral data the degradation impurity at RRT ~0.54 was confirmed as pregna-1, 4-diene-3, 20-dione, 21-(acetyloxy)-6, 9-difluoro-11, 16, 17-trihydroxy-(6 α , 11 β , 16 α)-.

3.2.10. Structure Elucidation of Degradation Impurity at RRT ~0.60

A LCMS study was carried to determine the m/z value of the major degradation product formed under acid hydrolysis using an Agilent 1100 series liquid chromatography system coupled with a 6400 series triple quadrupole mass spectrometer. The volatile mobile phase contained water, acetonitrile and acetic acid in a ratio of 60:40:1 (v/v/v) and the conditions were described in section 2.4. The molecular ion peak m/z value obtained for the degradation product resolving at 0.60 RRT is 454 $[(MH)]^+$, in ESI positive mode corresponding to a molecular weights of 452.5.

In 1H -NMR and ^{13}C -NMR spectra of new impurity shows chemical shift similarity with fluocinonide, except signals corresponding to C-21. In 1H -NMR the two protons on C-21 resonated as doublet of doublet at 4.20 & 4.70 ppm ($J = 19$ and 6 HZ) and the corresponding hydroxyl proton as triplet (which is a double doublet and appearing as triplet because of masking effect) at 4.95 ppm. Comparative 1H -NMR and ^{13}C -NMR spectral data for fluocinonide and RRT ~0.60 is given in **Table 3**.

The IR Spectra performed on dispersion KBr of RRT ~0.60 degradant show the following absorption bands

~3500 (broad OH Stretching), 2950-2850 (CH, CH₂ and CH₃ Stretching) 1720 (C = O Saturated keto group), 1660 (C = O Conjugation) 1630 and 1610 (C = C Stretching)

The elemental analysis data calculated for C₂₄H₃₀F₂O₆ (452.49): C, 63.70; H, 6.68; F, 8.4; O, 21.22 and found: C, 63.74; H, 6.64. From the above spectral data the degradation impurity at RRT ~0.60 was confirmed as pregna-1, 4-diene-3, 20-dione, 6, 9-difluoro-11, 21 dihydroxy-16, 17-[(1-methylethylidene) bis(oxy)]-, (6 α , 11 β , 16 α)-.

4. Conclusions

In this paper, a sensitive, specific, accurate, validated and well-defined stability indicating LC method for the determination of fluocinonide in the presence of degradation products and its process-related impurities was described. The behavior of fluocinonide under various stress conditions was studied, and the hydrolysis (acid and base) degradants were identified by LCMS and other spectral analysis presented. All of the degradation products and process impurities were well separated from the drug substance and drug product demonstrates the stability-indicating power of the method. The information presented in this study could be very useful for quality monitoring of drug substance and its dosage forms and be used to check drug quality during stability studies.

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