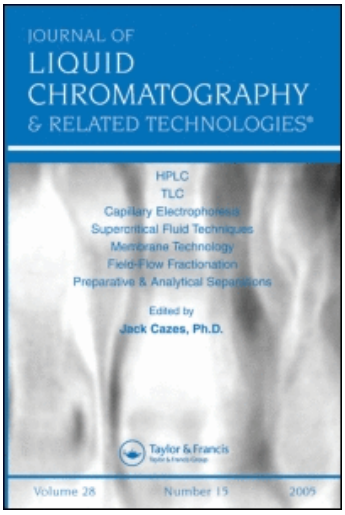


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# DEGRADATION STUDIES OF HIGHLY POTENT AND LIFE THREATENING HUMAN BIRTH DEFECT DRUG—LENALIDOMIDE BY HPLC AND LC-MS

**Nandan Srinivasan Raghu,[1,2] Y. Ramachandra Reddy,[1] V. Naresh,[2] V. Suryanarayana Rao,[1] and L. K. Ravindranath[1]**

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□ *Due to the potential toxicity of lenalidomide, a highly potent and severe life threatening human birth defect, a lot of precaution should to be taken in handling such kind of drugs.*

*Lenalidomide drug-excipient blend from the capsule pharmaceutical dosage form was subjected to different ICH prescribed stress conditions of thermal stress, pH hydrolysis, oxidation and photolysis. The drug was found to be stable only at photolysis and thermal stress, while it was extremely susceptible to other stressing conditions especially it showed extensive degradation under alkali conditions. For establishment of stability-indicating method, the rate of degradation in solutions in which different degradation products were formed were monitored at different time intervals, by using different concentrations of stressing reagents, and the separations was optimized by varying the LC conditions.*

*An acceptable separation was achieved through a multi-step gradient elution using an ACE® $C_{18}$, 150 × 4.6 mm i.d, 3 µm, stainless steel analytical column and a mobile phase comprising of 0.01 M phosphate buffer (pH, 2.0±0.1) as mobile phase-A, and a mixture of water and acetonitrile in the ratio of 200:800 (v/v) as mobile phase-B, with a flow rate and detection wavelength being 1.0 mL $min^{-1}$ and 220 nm respectively. The major degradation products appeared at relative retention times (RRT) of 0.75, 0.86, 0.96, 1.33, 1.52, 1.99, 2.04, 2.62 and 2.66 respectively.*

**Keywords** forced degradation studies, human birth defect, ICH, LC-MS, lenalidomide, pregnancy, method validation

## INTRODUCTION

The revised parent drug stability test guideline (ICH, Q1A (R2), 2003) by International Conference on Harmonization (ICH) requires that stress testing on the drug substance or drug product should be carried out to

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establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures.[1] It is suggested that stress testing should include the effect of temperature, humidity, light, oxidizing agents as well as susceptibility across a wide range of pH values. It is also recommended that analysis of stability samples should be done through the use of a validated stability indicating test methods.

Previously an HPLC assay method for lenalidomide on bulk samples (API) was reported.[2] However, there is no report yet on the development of an analytical method on the estimation and identification of degradants/impurities of lenalidomide in the presence of lenalidomide capsules (drug-product excipients).

Accordingly, the aims of the present study were to establish the inherent stability of lenalidomide in lenalidomide capsules through stress studies under a variety of ICH recommended test conditions[1,3–7] and to develop a stability indicating analytical method. The lenalidomide drug excipients blended from the capsule pharmaceutical dosage form underwent forced degradation to obtain the impurities profile and to demonstrate that the method could be employed during a stability study of the lenalidomide capsules. Also, the samples of forced degradation were subjected to LC-MS for identification of the possible degradants and to establish the inherent stability of the molecule.



Lenalidomide a thalidomide analogue is an immunomodulatory agent with anti-angiogenic and anti-neoplastic properties and is indicated in the treatment of multiple myeloma therapy. Lenalidomide and thalidomide are structurally related as they possess piperidindione and indoline moieties. They both have a asymmetric center and both are manufactured as racematic mixtures. Lenalidomide lacks the symmetrical indolindione of thalidomide and bears an amino function on its aromatic ring system, which contributes to its lower lipid solubility. Based upon the similarity in structure, one would predict that thalidomide and lenalidomide would be metabolized and degrade in a similar manner. The asymmetric carbon on each molecule bears acidic hydrogen and both molecules readily enolize. Imide hydrolysis and amide hydrolysis would explain the respective drug derived moieties formed by each other. Their degradative pathways, while apparently similar, have not resulted in any common degradation products in animals.[8,9]

The chemical name of lenalidomide is 3-(4-amino-1-oxo 1,3-dihydro-2-*H*-isoindol-2-yl) piperidine-2,6-dione. The empirical formula for lenalidomide is $C_{13}H_{13}N_3O_3$, and the gram molecular weight is 259.3. Lenalidomide is an off white to pale yellow solid powder. It is soluble in organic solvent/water mixtures, and buffered aqueous solvents. Lenalidomide is more soluble in organic solvents and low pH solutions. Solubility was significantly lower in less acidic buffers, ranging from about 0.4 to

0.5 mg $mL^{-1}$. Lenalidomide has an asymmetric carbon atom and can exist as the optically active forms S(−) and R(+), and is produced as a racemic mixture with a net optical rotation of zero.[8,9]

Revlimid®, the innovator reference product, is available in 5 mg, 10 mg, 15 mg, and 25 mg capsules for oral administration. Each capsule contains lenalidomide as the active ingredient and the following inactive ingredients: lactose anhydrous, microcrystalline cellulose, croscarmellose sodium, and magnesium stearate.[8,9]

## ''WARNINGS'': Special Prescribing Requirements for Those Who Take Lenalidomide[8,9]

Because of the potential toxicity and to avoid fetal exposure, lenalidomide is only available under special restricted distribution. Lenalidomide is an analogue of thalidomide. Thalidomide is a known human teratogen that causes severe life threatening human birth defects. If lenalidomide is taken during pregnancy, it may cause birth defects or death to an unborn baby. Females should be advised to avoid pregnancy while taking lenalidomide. Therefore, lenalidomide can be prescribed only by licensed prescribers who understand the potential risk of teratogenicity. If lenalidomide is used during pregnancy, effective contraception must be used by female patients of childbearing potential for at least 4 weeks before beginning lenalidomide therapy. Reliable contraception is indicated even where there has been a history of infertility, unless due to hysterectomy or because the patient has been postmenopausal naturally for at least 24 consecutive months. Two reliable forms of contraception must be used simultaneously unless continuous abstinence from heterosexual sexual contact is the chosen method. Females of childbearing potential should be referred to a qualified provider of contraceptive methods, if needed. Sexually mature females who have not undergone a hysterectomy, have not had a bilateral oophorectomy, or who have not been postmenopausal naturally for at least 24 consecutive months (i.e., who have had menses at some time in the preceding 24 consecutive months) are considered to be females of childbearing potential.

### *Dosing*[8,9]

The recommended starting dose of lenalidomide is 5 mg & 10 mg with water daily.

## EXPERIMENTAL

### Reference Substances, Chemicals, and Reagents

Lenalidomide API (standard, 99.9% purity), lenalidomide drug product excipients blended from the capsule pharmaceutical dosage form

and Impurity-C (Imp-C) used for the development study were kindly supplied by the Research Department of Dr. Reddy's Laboratories Limited, Hyderabad, India. Lactose anhydrous, microcrystalline cellulose, croscarmellose sodium, and magnesium stearate used were of USP-NF/Ph.Eur grade were also supplied by the Dr. Reddy's Laboratories Limited. Potassium hydrogen phosphate anhydrous, ortho-phosphoric acid, and acetonitrile were obtained from Merck (India). Sodium hydroxide was purchased from Ranbaxy Laboratories, acetic acid and hydrochloric acid was purchased from LOBA Chemie PVT. Ltd (India). Trifluro acetic acid (99% pure) was obtained from Acros organics, New Jersey, USA. All other reagents were of analytical reagent grade. High pure water was prepared by using Millipore Milli Q plus purification system.

## Instrumentation

### *HPLC Instrumentation*

The chromatographic separation was performed on an HPLC 1100 series, from Agilent Technologies, USA. The HPLC system consisted of an on-line degasser (G1379A), low pressure quaternary system delivery module (G1311A), auto injector and auto sampler (G1313A), column oven (G1316A), and UV-visible detector (G1314A). The output signal was monitored and processed using Empower software (Waters) on Pentium computer (Digital Equipment Co). Robustness and peak purity testing was done on another HPLC system equipped with a separation module (Waters 2695 model) and photo-diode array detector (Waters 2996 model).



### *Chromatographic Conditions*

An LC multi-step gradient method was developed for the separation of all possible related impurities, and degradants of lenalidomide. The mobile phase used for the analyses consists of 1.36 g of potassium hydrogen phosphate in 1000 mL of Milli-Q water (0.01 M), pH adjusted to $2.0 \pm 0.1$ with dilute ortho-phosphoric as mobile phase-A, and a mixture of water and acetonitrile in the ratio of 200:800 (v/v) as mobile phase-B. Both the mobile phase A and B were filtered through a 0.22 µm membrane filter before use. ACE® $C_{18}$, $150 \times 4.6$ mm i.d, 3 µm, stainless steel analytical column was used. The impurities were eluted according to the multi-step gradient by changing the % of (mobile phase-B) at different times, *T* (min)/% mobile phase B (v/v) = 0/0, 40/60, 50/80, 55/0, and 65/0. The mobile phase flow rate was maintained at 1.0 mL $min^{-1}$ and column eluent was monitored at 220 nm, at the column oven temperature of 25°C with the sample refrigerator temperature of 5°C. The sample was injected using a

10 µL fixed loop injector, and the chromatographic data was acquired for 65 min for each injection.

***Liquid Chromatography-Mass Spectrometry Conditions (LC-MS)***

Liquid chromatography-mass spectrometry (LC-MS) analysis was carried out using a MDS Sciex API, 4000QTRAP mass spectrometer (Applied biosystems, Foster city, CA, USA) coupled with Agilent HPLC 1100 series quaternary system delivery module. Analyst software (version 1.4.2) was used for data acquisition and data processing. LC-MS spectra were acquired from $m/z$ 100 to 1000 in 0.1 amu steps with 2.0 seconds dwell time. Forced degraded samples of drug product excipients were subjected to LC-MS analysis with the same chromatographic conditions as described above except, that the mobile phase-A was replaced with 0.1% Trifluro acetic acid.

## Analytical Procedures

***Extraction Solvent (Diluent), Standard and Test Solution***

Based upon the solubility and stability of lenalidomide, water and acetonitrile in the ratio of 500:500 v/v, respectively, was used as diluent for standard and test preparation.

***Preparation of Standard Solution (System Suitability Solution)***

Lenalidomide standard was accurately weighed and dissolved in the diluent to obtain a concentration of 0.5 µg $mL^{-1}$ (0.1% of the concentration of the lenalidomide in the test solution).

***Preparation of Test Solution***

Lenalidomide is available in multiple strengths as 5 mg, 10 mg, 15 mg, and 25 mg dosage capsules for oral administration. Common sample preparation methodology was adopted for each strength, so that it would ease the analyst at the quality control department, especially during stability analyses for each strength and at each stability station.

For 5 mg and 10 mg, twenty capsules were selected, while for 15 and 25 mg, ten capsules were taken. From each strength, their respective capsule shells were opened and the lenalidomide drug excipient blend from the capsules were pooled, and mixed well. From this pooled blend, the lenalidomide drug excipient blend was weighed for an amount equivalent to 50 mg of lenalidomide into a 100 mL volumetric flask, about 70 mL of diluent added, and the contents of the flask were kept on a rotary shaker for about 10 minutes and sonicated for about 5 minutes with intermediate

shaking (by maintaining the sonicator temperature below 25°C). Finally, the volume was completed with diluent and mixed well so as to obtain lenalidomide concentration of about 500 μg mL$^{-1}$. The solution was centrifuged in a tight enclosure for about 5 minutes at 3500 rpm and 10 μL of clear supernatant solution was injected directly on to the column.

## Quantitation

Peak areas were recorded for all the major peaks after blank (diluent) and placebo peaks correction. Respective peak areas were taken into account to quantitate (ICH Q3B (R2), 2006) the amounts in percentage as follows:

$$\frac{A \times C \times 100 \times N}{B \times D \times LC}$$

Where, $A$ is peak area obtained for any individual peak apart from the lenalidomide in the test solution; $B$ is the peak area obtained in the standard solution; $C$ is the concentration of standard solution; $D$ is the weight of lenalidomide drug excipient blend taken for the test solution; $N$ is the average weight of the net contents of each capsule obtained from each strength and $LC$ is labeled amount of lenalidomide in the lenalidomide capsules.



## Forced Degradation Studies (Stress Studies)

All the solutions for use in the forced degradation studies were prepared by adding small volumes of stressing reagents into the Lenalidomide drug product excipients blend sample. After the degradation these solutions were diluted with diluent to yield stated lenalidomide concentration of about 500 μg mL$^{-1}$. The stressing reagents, their concentrations, temperature, and the reaction time for forced degradation study were monitored so as to obtain a considerable degradation and to predict the intrinsic stability of lenalidomide in the capsule pharmaceutical dosage form. Conditions employed for performing the stress studies were as follows.

### *Hydrolytic Studies*

Acid degradation studies were performed by heating the solution of lenalidomide drug product excipients blend in 0.5 M HCl at 70°C on a water bath. The reaction for the degradation study was monitored at 2, 3.5, and 5 hours, respectively.

Acetic acid degradation study were performed by heating the solution of lenalidomide drug product excipients blend in 2 M acetic acid at 70°C

on a water bath. The reaction for the degradation study was monitored at 2 and 5 hours, respectively.

As the lenalidomide structure contains an amide functional group, which is easily susceptible for alkali degradation, the degradation study was performed by adding 0.05 M NaOH to the lenalidomide drug product excipients blend and the solution was injected immediately into the chromatographic system.

For the study in neutral conditions, lenalidomide drug product excipients blend solution in water was heated at 70°C for 1 hour prior to analysis.

### *Oxidation Studies*

Solutions for use in oxidation studies were prepared in 10% hydrogen peroxide and the resultant solution heated on a water bath at 70°C for 1.5 and 3 hours was injected prior to analysis.

### *Thermal Stress Studies*

Lenalidomide drug product excipients blend and placebo were exposed to dry heat of 80°C in a convention oven for 7 days and the samples were prepared as described earlier.



### *Photostability Studies*

Susceptibility of lenalidomide drug product excipients blend and placebo to light was studied (ICH Q1B, 1996). Lenalidomide drug product excipients blend and placebo for photo stability testing were placed in a light cabinet (Sanyo Photostability Chamber) and exposed to a white fluorescent lamp at the rate of 5 KiloLux/hour, for 10 days at 25°C that resulted in an overall illumination of 1200 KiloLux hours. Similarly, in the same photo stability cabinet, samples were exposed to near UV radiation having a spectral distribution from 320–400 nm, at the rate of 0.8 Watt/$m^2$/hour at 25°C for 250 hours, to achieve an overall illumination of $\geq$200 Watt/$m^2$/hour. Control samples, which were protected from light with aluminum foil were also placed in the same light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

## RESULTS AND DISCUSSION

### Degradation Behavior of Lenalidomide

HPLC studies of samples obtained on forced degradation studies of lenalidomide under different conditions and by using the above experimental conditions suggested the following degradation behavior.

A detailed % degradation against their corresponding HPLC RRT's and conditions, observed under forced degradation studies are depicted in Table 1.

## Hydrolytic Studies

### *Acid Degradation Studies*

The rate of hydrolysis in acid was very much slower as compared to that of alkali. The drug was found to be slightly unstable under 0.5 M HCl at 70°C. Drug degradation was associated with a rise in a major degradation product at an RRT of about 0.86 and 0.96, respectively (Figure 1a).

### *Acetic Acid Degradation Studies*

Interestingly, when we carried out lenalidomide degradation studies in the presence of 2 M acetic acid solution, some degradants of acetyl derivatives were observed, with a major degradant being at about 1.33 RRT (Figure 1b).



**TABLE 1** % Degradation Products Against their Corresponding HPLC RRT's and Conditions, Observed under Forced Degradation Studies

| | 10% Peroxide, 70°C | | 0.05 M NAOH, Bench Top | Water, 70°C | 0.5 M HCl, 70°C | | | 2 M Acetic Acid, 70°C | |
|---|---|---|---|---|---|---|---|---|---|
| RRT | 1.5 hrs | 3 hrs | 15 mins | 1 hr | 2 hrs | 3.5 hrs | 5 hrs | 2 hrs | 5 hrs |
| 0.636 | 0.52 | 1.25 | ND | ND | ND | ND | ND | ND | ND |
| 0.727 | 0.07 | 0.06 | ND | ND | ND | ND | ND | ND | ND |
| 0.752 | 0.29 | 0.53 | 30.64 | 0.82 | 0.44 | 0.7 | 0.85 | 0.11 | 0.36 |
| 0.789 | 0.77 | 1.69 | ND | ND | ND | ND | ND | ND | ND |
| 0.857 | 0.21 | 0.48 | 50.76 | 1.69 | 1.28 | 2.28 | 2.93 | 0.04 | 0.11 |
| 0.96 | ND | ND | 0.28 | ND | 0.33 | 1.17 | 2.27 | ND | ND |
| 1.185 | 0.05 | 0.42 | ND | ND | ND | ND | ND | ND | ND |
| 1.267 | 0.05 | 0.06 | ND | ND | ND | ND | ND | ND | ND |
| 1.318 | 0.06 | 0.06 | ND | ND | ND | ND | ND | ND | ND |
| 1.339 | 0.04 | 0.09 | ND | ND | ND | ND | ND | 0.52 | 1.47 |
| 1.435 | 0.07 | 0.12 | ND | ND | ND | ND | ND | ND | ND |
| 1.525 | 0.09 | 0.21 | ND | ND | ND | ND | ND | ND | ND |
| 1.977 | 0.75 | 0.71 | ND | ND | ND | ND | ND | ND | ND |
| 1.989 | 1.33 | 2.28 | ND | ND | ND | ND | ND | ND | ND |
| 2.04 | 0.05 | 0.16 | ND | ND | ND | ND | ND | 0.05 | 0.18 |
| 2.214 | 0.1 | 0.17 | ND | ND | ND | ND | ND | ND | ND |
| 2.269 | 0.11 | 0.19 | ND | ND | ND | ND | ND | ND | ND |
| 2.437 | 0.07 | 0.12 | ND | ND | ND | ND | ND | ND | ND |
| 2.623 | 1.26 | 0.86 | ND | ND | ND | ND | ND | ND | ND |
| 2.66 | 1.41 | 1.14 | ND | ND | ND | ND | ND | ND | ND |
| % Total degradation | 7.3 | 10.6 | 81.68 | 2.51 | 2.05 | 4.15 | 6.05 | 0.72 | 2.12 |

Where, ND represent Not detected at that corresponding RRT and degradation condition.

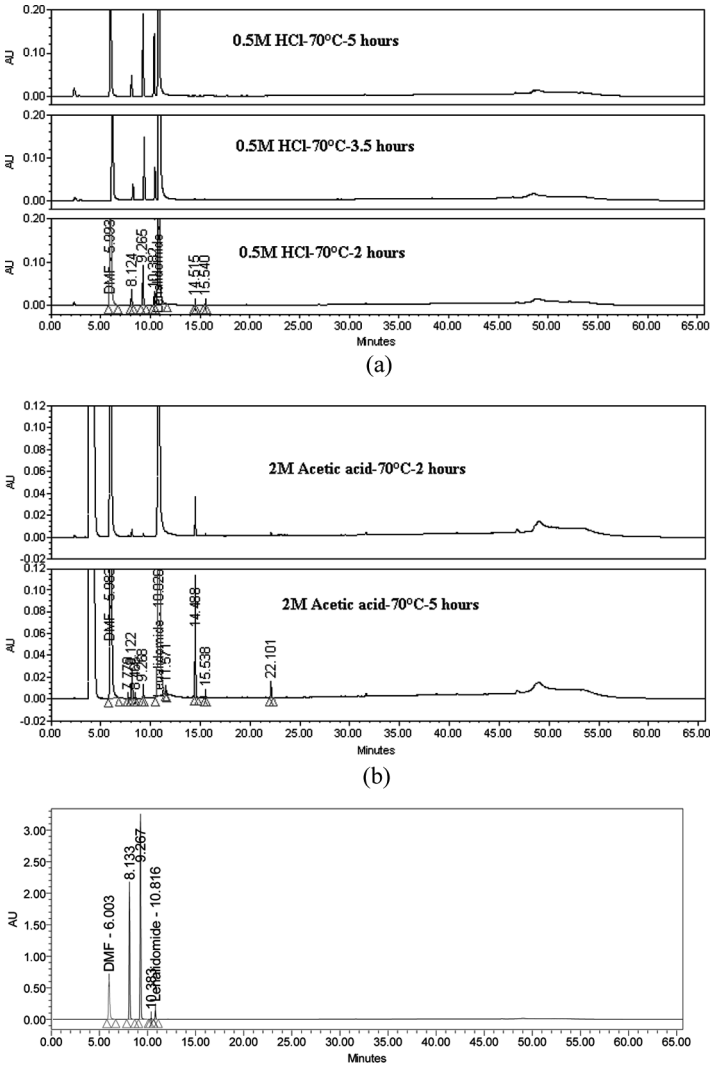
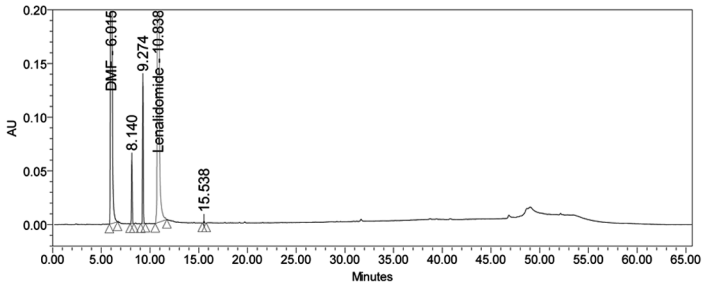
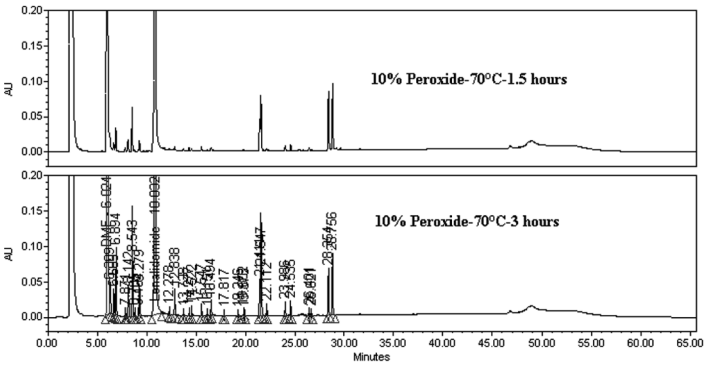


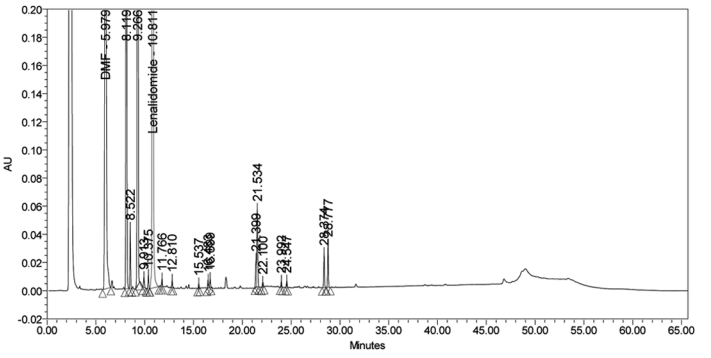

**FIGURE 1** (a) HPLC Chromatogram showing the separations of degradants observed under 0.5 M HCl degradation. (b) HPLC Chromatogram showing the separations of degradants observed under 2 M Acetic acid degradation. (c) HPLC Chromatogram showing the separations of degradants observed under 0.05 M NaOH degradation. (d) HPLC Chromatogram showing the separations of degradants observed under water degradation. (e) HPLC Chromatogram showing the separations of degradants observed under hydrogen peroxide degradation. (f) HPLC Chromatogram showing the separations of degradants observed under combination of Hydrogen peroxide and Base degradation. (g) HPLC Chromatogram of undegraded sample in the presence of diluent, when kept at 5°C sample temperature and analyzed at different time intervals.




(d)




(e)

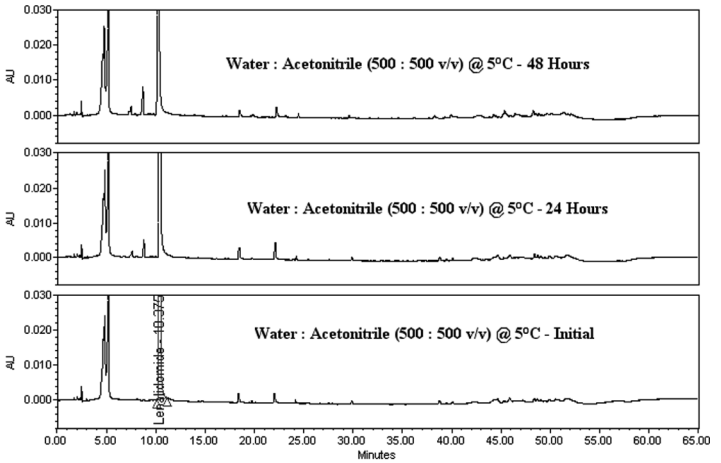



(f)

**FIGURE 1** Continued.

### *Base Degradation Studies*

As lenalidomide is easily susceptible for alkali degradation and the degradation study proved it to be highly unstable under basic pH conditions. The drug degraded so fast to an extent of about 82% with 31% and 51% as major unknown degradants at an RRT of about 0.75 and 0.86, respectively (Figure 1c).




(g)

**FIGURE 1** Continued.



### *Water Degradation Studies*

The degradation pattern observed under water degradation studies was found to be similar to that of alkali degradation, except with the low levels of degradation. The degradation was found to be about 0.82% and 1.7% as major unknown degradants at an RRT of about 0.75 and 0.86, respectively (Figure 1d).

## Oxidation Studies

The drug was found to be highly susceptible to hydrogen peroxide. Many degradants were observed during the study (Figure 1e, 1f). The details of the peroxide degradation pattern are depicted in Table 1.

## Photostability and Thermal Stress Studies

Thermal and light stress had no effect on lenalidomide. The drug was found to be highly stable with no major degradants in any of these conditions. The nature of degradation in light and dark was found to be similar, indicating that light had no effect on the degradation of the drug. The % impurities in all these samples were found to be <0.2%. A typical HPLC chromatogram of undegraded sample analyzed at different time intervals, when kept at 5°C sample temperature, is depicted in Figure 1g.

## Identification of Major Degradants by LC-MS

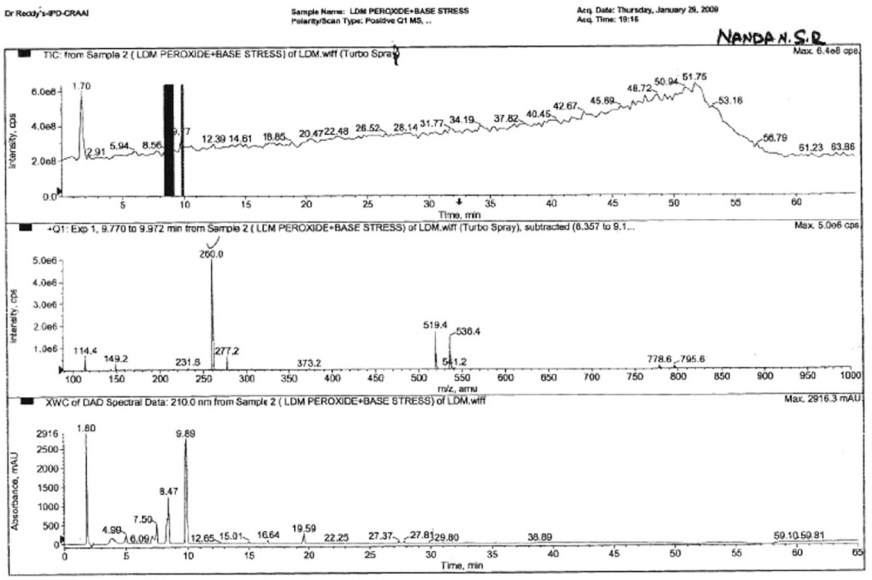
Mass chromatograms in the positive mode for the lenalidomide drug and degradation products are depicted in Figure 2a to Figure 2h. Evidently the m/z value of 260.0 for lenalidomide corresponded to its literature reported[8,9] molecular weight of 259.3, thus validating the output of the mass spectrometer. Based on the molecular mass of degradants, some of the possible structures of these degradants are depicted in Figure 2i. The m/z values obtained for the degradation products resolving at RRT's 0.75, 0.86, 0.96, 1.33, 1.52, 1.99, 2.04, 2.62, and 2.66 were 261.2, 261.2, 279.4, 302.2, 130.2, 290.0, 294.2, 538.0, and 568.2, respectively. Despite that its standards are not available, the possibility of the proposed degradants were found to be matching with those provided from a literature report (NDANo: 21-880).[8]

## Chromatographic Separation Studies (Method Development)

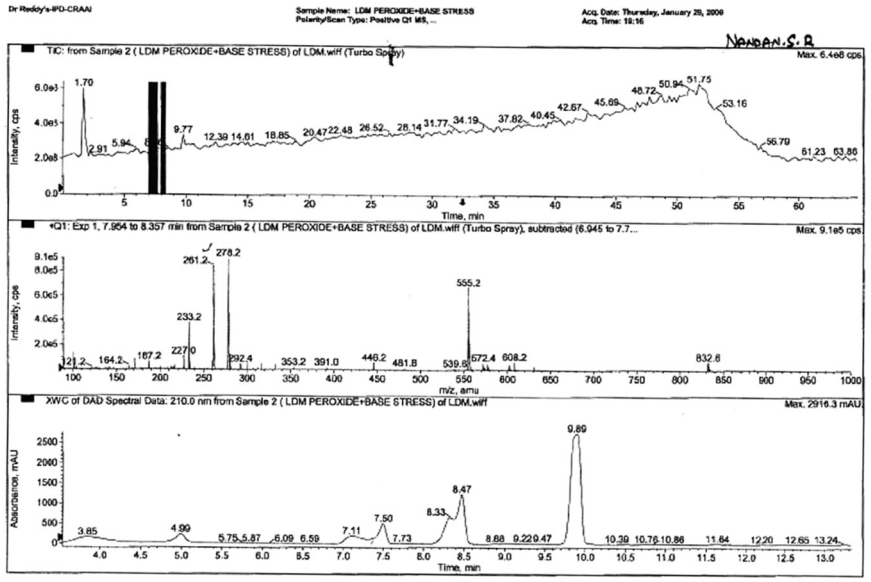


Previously an HPLC assay method for lenalidomide on API has been reported.[2] The adaptation of the same method for the capsule pharmaceutical dosage formulations was found to be unsuitable because of the following reasons. (1) The isocratic chromatographic conditions adopted in the reported method was found to be poor enough to separate all the degradants observed during stress studies. Overlaps of degradants peaks with lenalidomide were observed because of the early retention time for lenalidomide. (2) The study was performed only on API, hence placebo interference study was not reported. Placebo excipients interferences, especially at lower wavelength of 220 nm necessary for lenalidomide and its impurities, made it unsuitable for estimation of impurities of lenalidomide in capsule pharmaceutical dosage forms. (3) The sample concentration for lenalidomide as reported in the literature was not sensitive enough to achieve the required LOQ for impurities as per the ICH.[7] (4) The elution strength of mobile phase was very low. Many non-polar degradants observed during the stress studies were not eluted, even after extending the analytical run time for 50 min (twice the reported method), as a result of which it led to carry over in the next sample analyses. (5) The reported method study was mainly focused on the assay of lenalidomide in lenalidomide API. Hence, no study on the estimation of % impurities or % degradants of lenalidomide in the presence of lenalidomide drug excipients blend and their impurities identification is available.

Considering the above facts, and since no analytical method has ever been reported in the literature on lenalidomide drug excipients capsule dosage form, it was very important for us to propose a new suitable stability

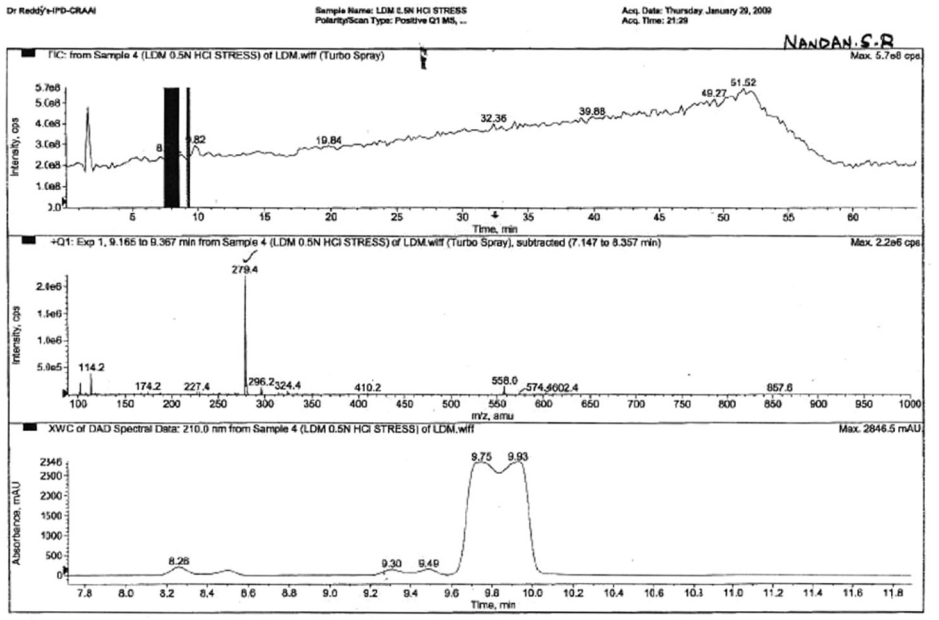



(a)

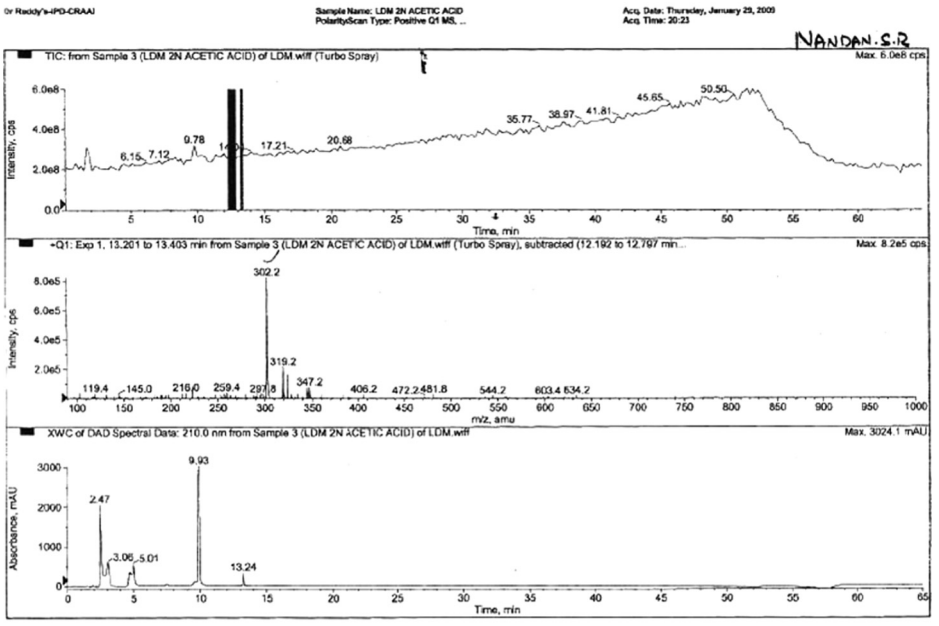



(b)

**FIGURE 2** (a) An LC-MS Chromatogram of Lenalidomide with m/z value of 260.0. (b) An LC-MS Chromatogram of degradant at RRT of 0.75 with m/z value of 261.2. (c) An LC-MS Chromatogram of degradant at RRT of 0.96 with m/z value of 279.4. (d) An LC-MS Chromatogram of degradant at RRT of 1.33 with m/z value of 302.2. (e) An LC-MS Chromatogram of degradant at RRT of 1.52 with m/z value of 130.2. (f) An LC-MS Chromatogram of degradant at RRT of 1.99 with m/z value of 290.0. (g) An LC-MS Chromatogram of degradant at RRT of 2.62 with m/z value of 538.0. (h) An LC-MS Chromatogram of degradant at RRT of 2.66 with m/z value of 568.2. (i) Schematic representation of probable structure of the lenalidomide and degradants observed under forced degradation studies, along with their molecular mass (m/z) from LC-MS.

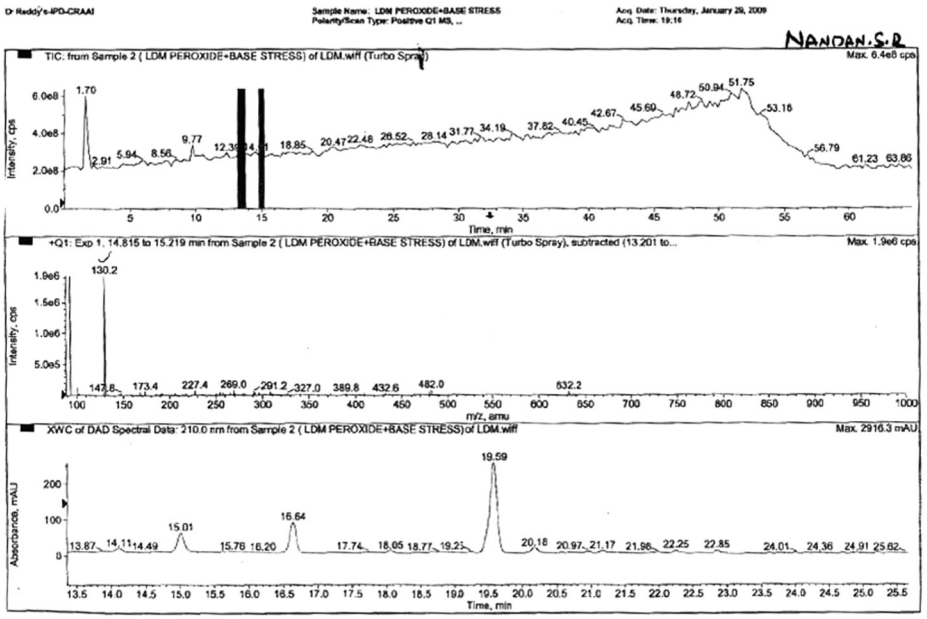



(c)

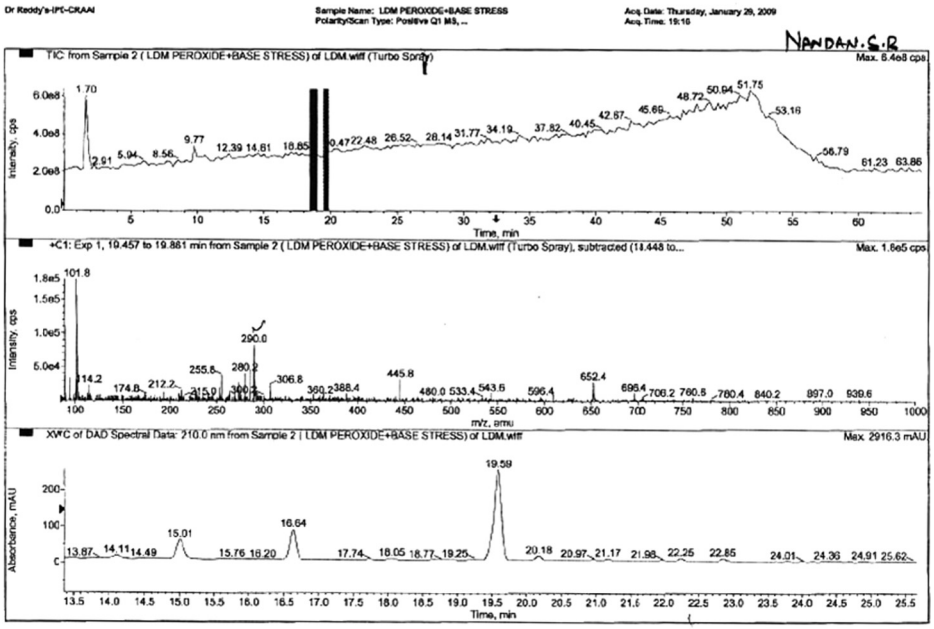



(d)

**FIGURE 2** Continued.

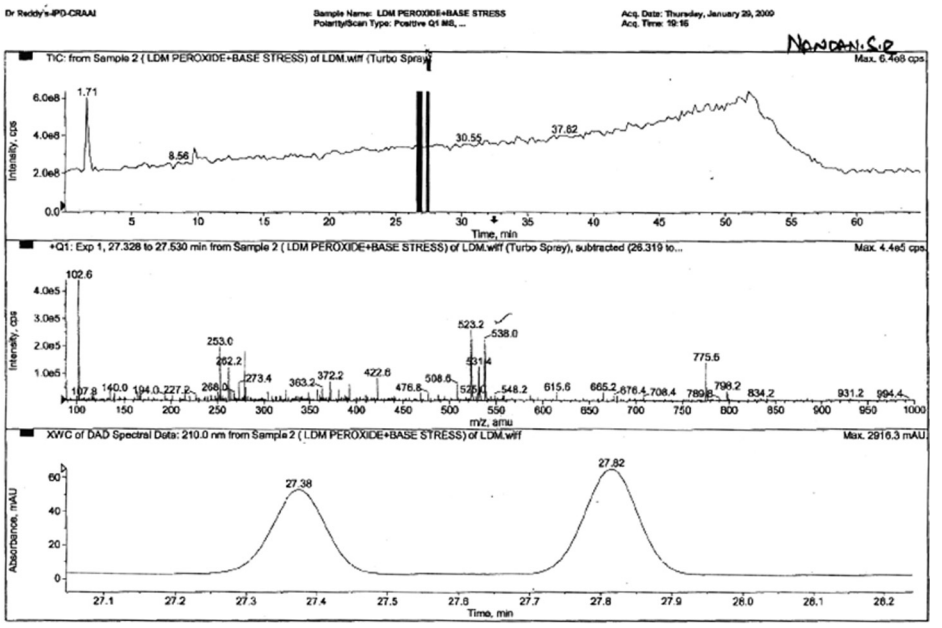



(e)

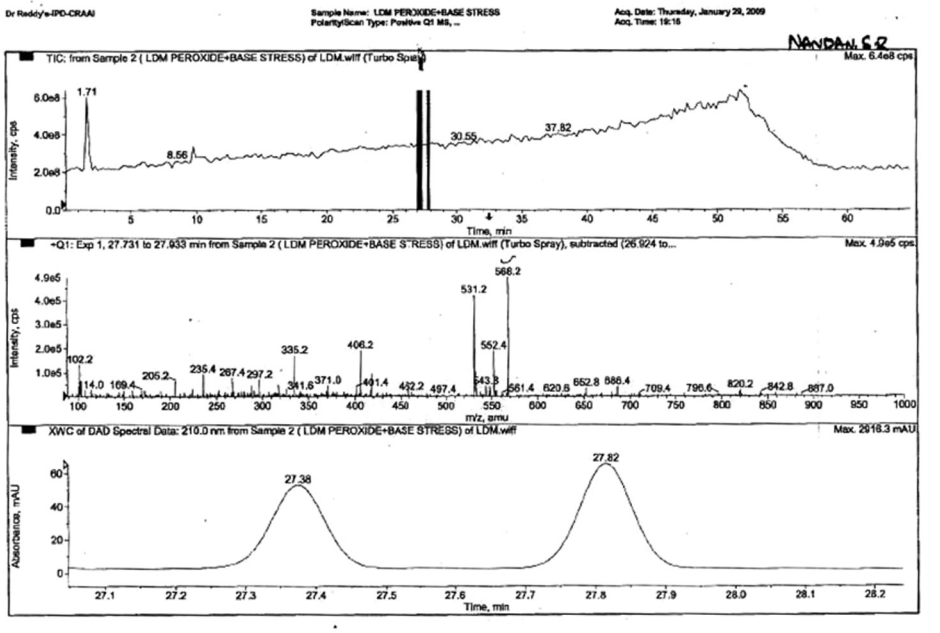



(f)

**FIGURE 2** Continued.

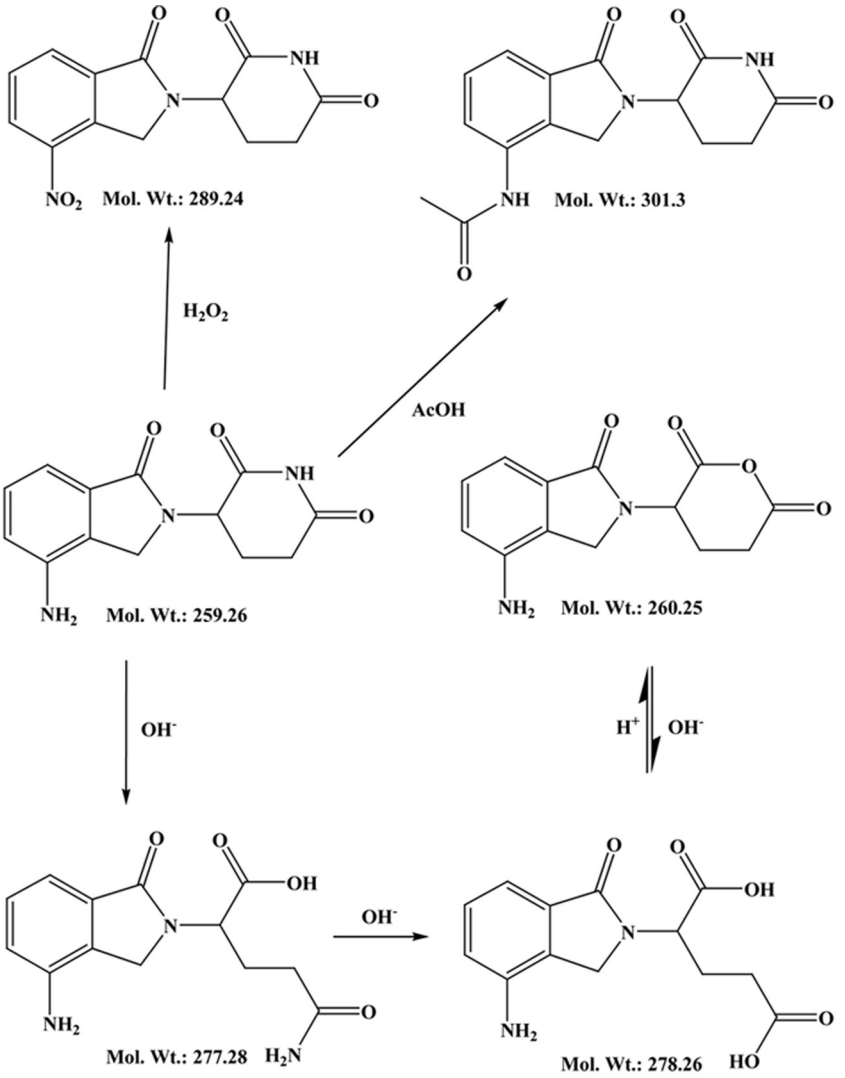



(g)




(h)

**FIGURE 2** Continued.




(i)

**FIGURE 2** Continued.

indicating method for estimation of impurities of lenalidomide in capsule dosage pharmaceutical form.

The proposed method took several gradient optimization studies before we could propose the final chromatographic conditions, as described in the earlier section. The gradient optimization study was performed so that all the peaks that were observed under degradation studies, especially in the peroxide conditions, are well resolved from each other, and about 60% of

organic modifier was introduced into the gradient such that, all the late eluting non-polar impurities are well eluted without any carry over into the next chromatographic run. An analytical 3 µm column was used during the study as compared to 5 µm, because of good separations. All the separations as observed under all the forced degradation conditions were achieved on an ACE® $C_{18}$, 150 × 4.6 mm i.d., 3 µm analytical column. The similar chromatographic analytical method conditions were extended to LC-MS for impurities identification, except that mobile phase-A was replaced with trifluoroacetic acid because of its suitability for LC-MS.

## Analytical Method Validation

The proposed test method was validated following the requirements of International conference on Harmonization (ICH) guidelines.[10,11] Parameters like specificity, linearity, precision, accuracy, robustness, and system suitability were examined.



## Specificity

There were no interferences due to placebo (excipients used in the capsule, without API) and sample diluent at the retention times of lenalidomide and its related impurities. When the degradation of lenalidomide was induced by hydrolytic, oxidation, light exposure, and thermal stress, the HPLC chromatograms of the resulting solutions showed the method is stability indicating in nature. The HPLC chromatograms (Figure 1a to Figure 1g) recorded after degradation showed well resolved peaks for almost all degradants and impurities. These degradation products showed also a significant resolution from lenalidomide under all the stress conditions. Homogeneity of lenalidomide and Imp-C were established using a PDA detector. All related impurities were well separated under all the forced degradation studies conditions employed, and purity angle was found to be less than purity threshold (for Waters Empower software) for lenalidomide. Apart from the peaks homogeneity, the DAD spectrum for lenalidomide was compared against its standard spectrum. Identity for the lenalidomide was performed by comparing its DAD spectrum and purity plots along with those of standards and was found to be matching.

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

The limits of detection and Quantitation were evaluated by determining the concentrations by serial dilutions of Imp-C and lenalidomide in the presence of placebo in order to obtain signal to noise ratios of ~3:1

**TABLE 2** Results of Precision and Accuracy at LOQ Level

| | Imp-C | | | | Lenalidomide | | | |
|---|---|---|---|---|---|---|---|---|
| Replicate # | RT (mins) | Area | % Imp-C | % Recovery | RT (mins) | Area | % Lena | % Recovery |
| 1 | 14.8 | 7067 | 0.018 | 90.0 | 10.1 | 7100 | 0.019 | 95.0 |
| 2 | 14.9 | 7234 | 0.019 | 95.0 | 10.2 | 7157 | 0.019 | 95.0 |
| 3 | 14.8 | 7467 | 0.019 | 95.0 | 10.1 | 7268 | 0.019 | 95.0 |
| 4 | 14.8 | 6805 | 0.018 | 90.0 | 10.2 | 6940 | 0.018 | 90.0 |
| 5 | 14.9 | 7250 | 0.019 | 95.0 | 10.1 | 7373 | 0.019 | 95.0 |
| 6 | 14.8 | 7761 | 0.020 | 100.0 | 10.2 | 7560 | 0.020 | 100.0 |
| Average | 14.8 | 7264 | 0.019 | 94.2 | 10.2 | 7233 | 0.019 | 95.0 |
| Std dev | 0.05 | 328.3 | 0.001 | 3.8 | 0.1 | 217.7 | 0.001 | 3.2 |
| % RSD | 0.3 | 4.5 | 4.0 | 4.0 | 0.5 | 3.0 | 3.3 | 3.3 |

for LOD and ~10:1 for LOQ. From this study, the limit of detection (LOD) for Imp-C and lenalidomide was found to be 0.04 and 0.05 $\mu g\ mL^{-1}$, while limit of Quantification (LOQ) was found to be 0.09 and 0.11 $\mu g\,mL^{-1}$, respectively. For precision and accuracy of the LOQ level, the samples were prepared six times by spiking Imp-C and lenalidomide at about limit of quantification level in the presence of placebo and were injected into the HPLC. The % RSD for precision and % recovery for accuracy for both Imp-C and lenalidomide was calculated. The results of LOQ for precision and accuracy are tabulated in Table 2.



## Response Linearity

The detector response linearity parameter of the curve for Imp-C and lenalidomide was determined. The mixed seven standard solutions containing Imp-C and lenalidomide in the concentration range of LOQ to about 1.0% (W.R.T 500 $\mu g\,mL^{-1}$ lenalidomide test concentration) were prepared and injected into the chromatographic system. The results of correlation coefficient, values of Slope (*b*) and Intercept (*a*) for both Imp-C and lenalidomide as a function of concentration $\mu g\,mL^{-1}$ (*X-axis*) and detector peak (area) response (*Y-axis*) is depicted in Table 3. In this simultaneous determination, the linear regression was found to be good over the concentrations range mentioned (Figures 3a, 3b).

## Precision

### *System Precision*

Instrumental precision for lenalidomide at 0.5 $\mu g\,mL^{-1}$(0.1% of the concentration of the lenalidomide in the test solution) as system suitability was determined by analyzing six replicate injections on different systems

**TABLE 3** Results of Detector Response Linearity

| | Imp-C | | | Lenalidomide | | | |
|---|---|---|---|---|---|---|---|
| Dilution # | $\mu g\,mL^{-1}$ | % Level | Peak Area | Dilution # | $\mu g\,mL^{-1}$ | % Level | Peak Area |
| 1 | 0.09 | 0.018 | 7100 | 1 | 0.11 | 0.022 | 7342 |
| 2 | 0.632 | 0.126 | 51000 | 2 | 0.639 | 0.128 | 53000 |
| 3 | 1.263 | 0.253 | 99746 | 3 | 1.279 | 0.256 | 97746 |
| 4 | 1.974 | 0.395 | 157216 | 4 | 1.998 | 0.400 | 160216 |
| 5 | 2.527 | 0.505 | 205213 | 5 | 2.557 | 0.511 | 215213 |
| 6 | 3.553 | 0.711 | 281234 | 6 | 3.596 | 0.719 | 295234 |
| 7 | 5.527 | 1.105 | 440897 | 7 | 5.594 | 1.119 | 439897 |
| Correlation coefficient | | | 0.9999 | Correlation coefficient | | | 0.9991 |
| Slope (*b*) | | | 79693 | Slope (*b*) | | | 79635 |
| Y-Intercept (*a*) | | | 269.59 | Y-Intercept (*a*) | | | 1780.37 |
| % Y-Intercept | | | 0.13 | % Y-Intercept | | | 0.83 |



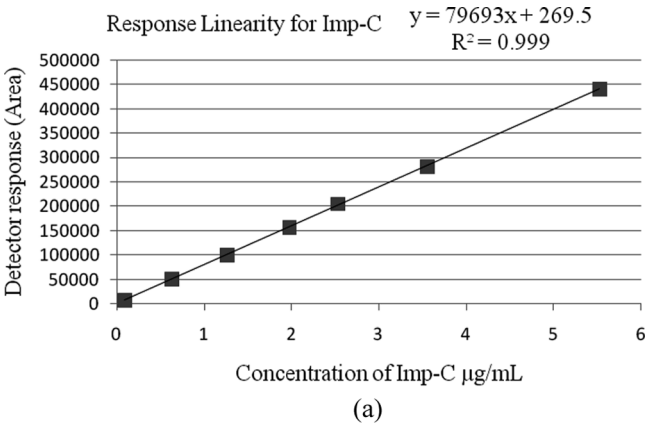
and different days and the relative standard deviation was found to be 1.4% and 3.3%, respectively. The results of system suitability and system precision are depicted in Table 4.

### *Method Precision (Repeatability) and Intermediate Precision*

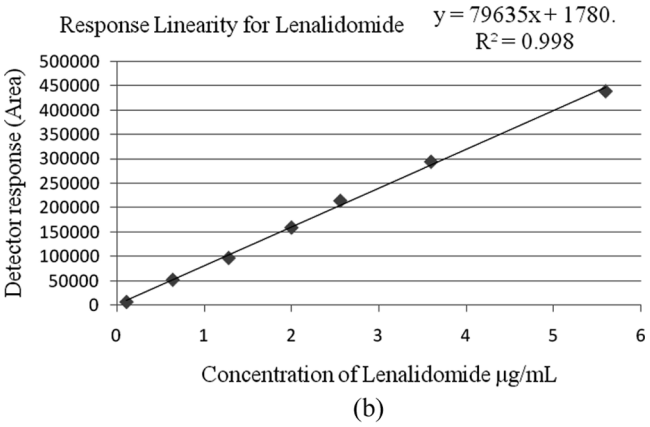
Method precision or intra-day precision was performed by spiking Imp-C at $2.5\,\mu g\,mL^{-1}$ level (0.50% level of the concentration of the lenalidomide in the test solution, as specification limit) in the presence of lenalidomide drug excipient blend samples. Six replicate ($n = 6$) solutions were prepared and each solution was injected in duplicate under the same conditions and mean value of peak area response was considered. Intermediate precision (inter-day precision) was performed by analyzing the study using different instruments, analysts, columns, and six different samples at the stated concentration. The results of repeatability and intermediate precision experiments are shown in Table 5. The results of Table 5 reveals that, the developed method was found to be precise as the RSD values which was <5.0% on both the variations, respectively.

### *Accuracy (Recovery Test)*

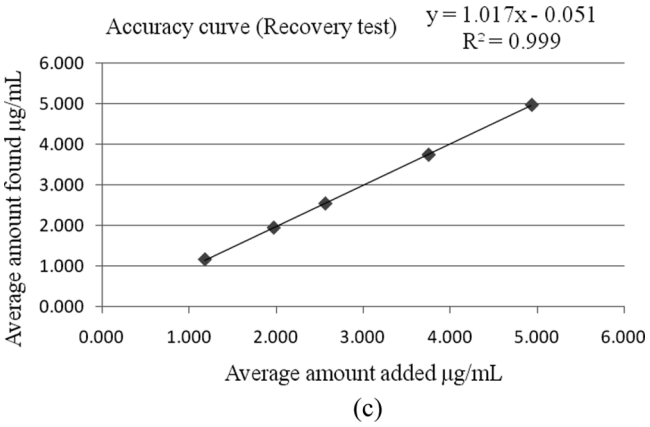
The accuracy of the method was evaluated by the recovery studies, which were carried out by spiking the Imp-C at different concentrations in the presence of lenalidomide drug excipient blend samples in the concentration range from $1.2\,\mu g\,mL^{-1}$ to $4.9\,\mu g\,mL^{-1}$ (50% to 200% of 0.50% level of the concentration of the lenalidomide in the test solution). Three samples were prepared for each concentration. The results of the spiked




(a)




(b)




(c)

**FIGURE 3** (a) Response linearity graph for Imp-C. (b) Response linearity graph for lenalidomide. (c) Graph for accuracy curve (Recovery test, linearity of Test method for Imp-C).

**TABLE 4** System Suitability Parameters for Lenalidomide

| Intra-Day | | | | Inter-Day | | | |
|---|---|---|---|---|---|---|---|
| Injection # | Peak Area | USP Theoretical Plates | USP Tailing Factor | Injection # | Peak Area | USP Theoretical Plates | USP Tailing Factor |
| 1 | 38919 | 10576 | 1.2 | 1 | 41919 | 12500 | 1.1 |
| 2 | 37218 | 10611 | 1.1 | 2 | 40218 | 11634 | 1.2 |
| 3 | 37989 | 10723 | 1.1 | 3 | 39989 | 12709 | 1.1 |
| 4 | 38200 | 11234 | 1.2 | 4 | 43200 | 10897 | 1.2 |
| 5 | 37892 | 12345 | 1.2 | 5 | 40892 | 11098 | 1.1 |
| 6 | 37999 | 10987 | 1.2 | 6 | 42999 | 11238 | 1.2 |
| Average | 38036 | 11079 | 1.2 | Average | 41536 | 11679 | 1.2 |
| Std dev | 547.7 | — | — | Std dev | 1385.7 | — | — |
| % RSD | 1.4 | — | — | % RSD | 3.3 | — | — |

concentrations for the related impurity are depicted in Table 6. The results of accuracy revealed that, the average recovery at each level and for each Imp-C was within $100 \pm 5\%$ with RSD at each level being $\leq 5\%$.



### *Accuracy Curve*

The accuracy curve (linearity of test method) was established by plotting the values of average amount added for Imp-C at each level in $\mu g\,mL^{-1}$ on *X*-axis, and average amount found for Imp-C at each level in $\mu g\,mL^{-1}$ on *Y*-axis as determined from accuracy section. The correlation coefficient ($R^2$), slope, and *Y* intercept values are depicted in Figure 3c, respectively.

**TABLE 5** Repeatability and Intermediate Precision Data Evaluated through Intra-Day and Inter-Day Studies

| | Intra-Day Precision | | | | | Inter-Day Precision | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dilution # | [z]μg $mL^{-1}$ | % Level | Peak Area | % Imp-C | % Rec | [z]μg $mL^{-1}$ | % Level | Peak Area | % Imp-C | % Rec |
| 1 | 2.55 | 0.51 | 209234 | 0.53 | 103.92 | 2.45 | 0.49 | 202156 | 0.49 | 100.00 |
| 2 | 2.55 | 0.51 | 202908 | 0.51 | 100.00 | 2.45 | 0.49 | 199765 | 0.48 | 97.96 |
| 3 | 2.55 | 0.51 | 206123 | 0.50 | 98.04 | 2.45 | 0.49 | 204356 | 0.49 | 100.00 |
| 4 | 2.55 | 0.51 | 208001 | 0.53 | 103.92 | 2.45 | 0.49 | 193896 | 0.48 | 97.96 |
| 5 | 2.55 | 0.51 | 209112 | 0.50 | 98.04 | 2.45 | 0.49 | 203452 | 0.50 | 102.04 |
| 6 | 2.55 | 0.51 | 207569 | 0.53 | 103.92 | 2.45 | 0.49 | 201569 | 0.48 | 97.96 |
| Average | | | 207158 | 0.52 | 101.31 | Average | | 200866 | 0.49 | 99.32 |
| Std dev | | | 2372.39 | 0.02 | 2.95 | Std dev | | 3763.48 | 0.01 | 1.67 |
| % RSD | | | 1.1 | 2.9 | 2.9 | % RSD | | 1.9 | 1.7 | 1.7 |

[z]Spiked concentration.

**TABLE 6** Accuracy (Recovery Test)

| Replicate # | Spike Level | Amount Added $\mu g\,mL^{-1}$ | Peak Area | Amount Found $\mu g\,mL^{-1}$ | % Rec | Average | Std Dev | % RSD |
|---|---|---|---|---|---|---|---|---|
| T1 | 50% | 1.184 | 90678 | 1.191 | 100.59 | 98.65 | 1.86 | 1.89 |
| T2 | 50% | 1.184 | 88765 | 1.166 | 98.48 | | | |
| T3 | 50% | 1.184 | 87345 | 1.147 | 96.88 | | | |
| T1 | 75% | 1.974 | 143498 | 1.884 | 95.44 | 98.84 | 3.06 | 3.09 |
| T2 | 75% | 1.974 | 149876 | 1.968 | 99.70 | | | |
| T3 | 75% | 1.974 | 152349 | 2.001 | 101.37 | | | |
| T1 | 100% | 2.566 | 198345 | 2.605 | 101.52 | 99.33 | 2.06 | 2.08 |
| T2 | 100% | 2.566 | 190345 | 2.500 | 97.43 | | | |
| T3 | 100% | 2.566 | 193456 | 2.541 | 99.03 | | | |
| T1 | 150% | 3.751 | 292345 | 3.839 | 102.35 | 100.04 | 2.98 | 2.98 |
| T2 | 150% | 3.751 | 288765 | 3.792 | 101.09 | | | |
| T3 | 150% | 3.751 | 276123 | 3.626 | 96.67 | | | |
| T1 | 200% | 4.935 | 388123 | 5.097 | 103.28 | 100.96 | 2.13 | 2.11 |
| T2 | 200% | 4.935 | 377689 | 4.960 | 100.51 | | | |
| T3 | 200% | 4.935 | 372398 | 4.890 | 99.09 | | | |



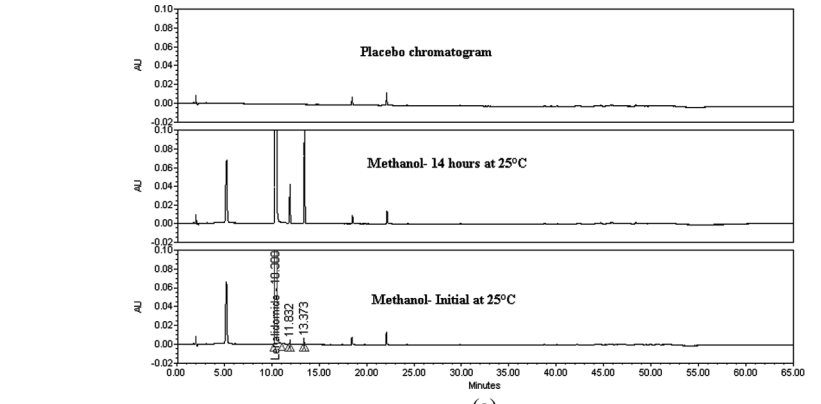
## Stability of Analytical Solution

The choice of diluent as described in the earlier section was based on the stability of lenalidomide. It was observed that either methanol or its combination with water, degraded lenalidomide very badly. So methanol was replaced with acetonitrile. Eventually water and acetonitrile was used as diluent. Even though the degradation of lenalidomide in the presence of water and acetonitrile as the diluent in the ratio of 500:500 v/v, respectively, minimized the degradation, still the solution was found to be unstable at 25°C. However, when the same solution was refrigerated at 5°C sample temperature, the degradation was controlled. A detailed pattern of degradation of lenalidomide in the presence of different diluents is depicted in the Figures 1g, 4a, 4b, 4c, and 4d. On the basis of Figures 1g, 4a, 4b, 4c, and 4d results, it was recommended to use 5°C as sample temperature during the analyses.

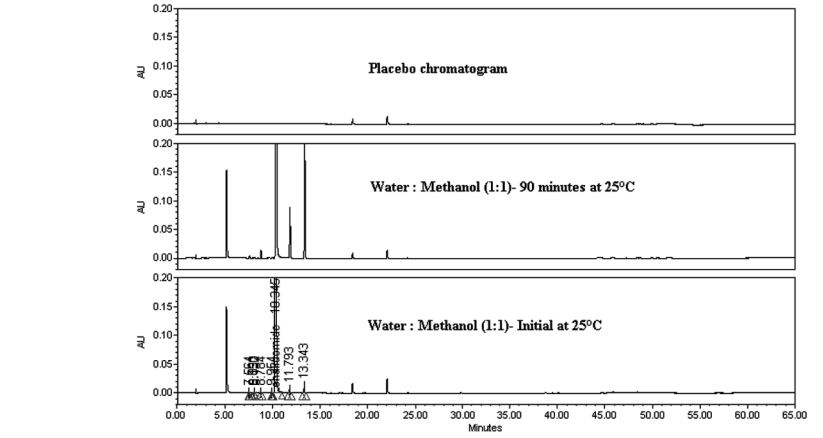
## Robustness

Robustness of the proposed method was performed by keeping chromatographic conditions constant with the following differences:

1. Increasing the column oven temperature from 20°C to 30°C.
2. Change in the flow rate of mobile phase from 0.8 mL to 1.0 $mL\,min^{-1}$.
3. Using another column of the same brand of different Lot. (ACE® $C_{18}$, $150 \times 4.6$ mm i.d., 3 µm).




(a)

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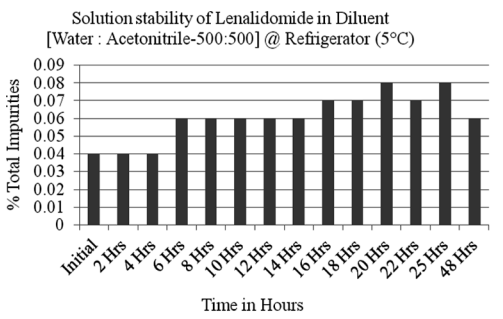
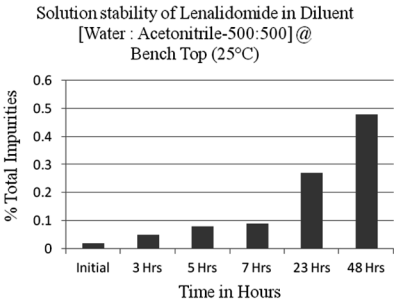



(c)




(d)

**FIGURE 4** (a) HPLC Chromatogram showing the effect of methanol diluent on lenalidomide, when analyzed at different time intervals, when kept at 25°C sample temperature. (b) HPLC Chromatogram showing the effect of water: methanol diluent on lenalidomide, when analyzed at different time intervals, when kept at 25°C sample temperature. (c) Results of solution stability of lenalidomide in the presence of diluent, when stored at 5°C sample temperature and analyzed at different time intervals. (d) Results of solution stability of Lenalidomide in the presence of diluent, when stored at 25°C sample temperature and analyzed at different time intervals.

4. Change in the composition of mobile phase B.
5. Change in the pH of the mobile phase from 1.8 to 2.2.

For each change, the standard solution was injected six times. System suitability parameters like peak symmetry, theoretical plates, and relative standard deviation were recorded for lenalidomide and found to remain unaffected. Also, the degraded samples were injected for each change and a baseline separation was achieved for all the samples. The method was found to be robust for the above variations without any interference.

### Analysis of Stability Samples

Lenalidomide capsule stability samples were subjected to the accelerated stability conditions of 60°C for 3 months. The test solutions subjected to HPLC analysis showed no major degradants with % of total impurities <0.2% when compared against the degradation studies. Peak identity for lenalidomide samples were found to be matching with those of the standard DAD spectrum, and peak homogeneity was proven through a PDA detector.



## CONCLUSIONS

This paper reports for the first time a novel method to quantitate related impurities of lenalidomide in the presence of lenalidomide capsules finished dosage form by RP-HPLC. The results of forced degradation studies undertaken according to the ICH guidelines reveal that the method is selective and stability indicating. The proposed HPLC method has the ability to separate lenalidomide from their degradation products and related impurities, and excipients found in the capsule dosage form and, therefore, can be applied to the analysis of samples for quality control. The method is rapid, direct, specific, accurate, precise, stability indicating, and validated for the routine analysis in the finished dosage form. The method may also be extended to evaluate active drug substances.

It was possible in this study to develop a stability indicating method for estimation of impurities and degradants by subjecting the drug to the ICH recommended stress conditions. The method proved to be simple, accurate, precise, and specific. The method was easily transferable to LC-MS. The stress studies and subsequent LC-MS analyses showed the intrinsic stability of lenalidomide and the possible degradation pathways under hydrolytic and oxidative conditions. An added finding was that the lenalidomide drug excipient blend was practically stable to dry heat and photolysis. The same analytical method can be adopted (as the sample

preparation and concentrations are identical) for the multiple strengths of lenalidomide, which is the added advantage of this paper.

Further, this paper also emphasized that many precautions need to be taken while handling a highly potent and severe life threatening human birth defects drug.

## ACKNOWLEDGMENTS

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