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Investigation of the Chemical Stability of Lenalidomide in Methanol/Ethanol Solvents Using RP-HPLC-UV and LC-MS

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ABSTRACT

Lenalidomide is a heterocyclic drug used for the treatment of Myelodysplastic syndrome. The current research focuses on the structural elucidation of new degradants that are formed unexpectedly upon storage of lenalidomide in methanol, followed by proposing their corresponding formation mechanism. The proposed structures of the degradants are relatively stable in which two tetrahedral intermediates are resulted from nucleophilic addition of methanol to the carbon of the carbonyl group of imide ring. Methanol molecules, as a solvent, may contribute in stabilizing the intermediate via hydrogen bond formation with it. These degradants were found abundant in lenalidomide/ methanol solution. Hence, the toxicological evaluation of them is crucial.

Keywords: Lenalidomide, Degradants, RP-HPLC, Mass spectrometry.

INTRODUCTION

Lenalidomide [3-(4-amino-1-oxo 1,3-dihydro-2*H*-isoindol-2-yl) piperidine-2,6-dione] (Fig. 1) is a heterocyclic drug which is used for the treatment of Myelodysplastic Syndrome (MDS) [1,2]. Lenalidomide is a potent thalidomide analog showed fewer adverse effects than the potent drug thalidomide. Lenalidomide displayed promising results in phase II trials towards myelofibrosis and myelofibrosis with myeloid metaplasia[3]. Consequently and based on the success of the clinical studies, lenalidomide was approved by the US Food and Drug Administration (US-FDA) and registered by Celgene Corporation (New Jersey, USA) under the commercial name Revlimid® [4].

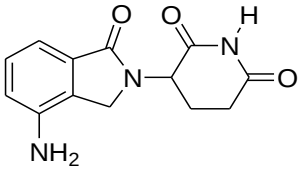

Fig. 1. Chemical structure of lenalidomide

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Identification of significant degradants and impurities in the active pharmaceutical ingredient (API) is mandatory by many regulatory authorities [5] and should be well-established since these impurities may affect the safety and efficacy of APIs [6,7]. Furthermore, some of the unidentified degradants could be hazardous and toxic even if they present in low quantities in the finished pharmaceutical formula [8,9]. Therefore, chemical structures of these substances should be well characterized. Few papers were reported for the determination of lenalidomide related impurities/degradants in the raw material or in the finished product. Lenalidomide-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 separations and characterization of components were achieved through a multi-step gradient elution using an HPLC with spectrophotometric and Mass techniques [10].

A new chromatographic method was established for the determination of lenalidomide and its related substances in capsules using Sunfire C-18 column with 85:15 v/v ratio of mobile phases A (mixture of phosphoric acid buffer and 1-octane sulphonic acid sodium salt) and B (55: 45 v/v ratio of methanol and acetonitrile) at 210 nm wavelength. The degradation studies were conducted using 0.1 M HCl, 0.1 M NaOH, 1% (v/v) H_2O_2 solutions, UV at 254 nm, Sun light, and heat to 60ºC. No significant degradation of lenalidomide was detected [11].

In continuation to our recent research in the respect of structural determination of new degradation products in various pharmaceutical ingredients [12–15], the current research is focusing on the investigation of the chemical stability of lenalidomide starting material in methanol and ethanol followed by structural elucidation of new degradants that are formed unexpectedly upon storage of lenalidomide in the former organic solvents, followed by proposing their corresponding formation mechanisms. Methanol and ethanol (protic organic solvents) are highly employed in the literature for preparing various lenalidomide solutions. Establishment a new analytical method for the assay determination of lenalidomide is not the scope of the present work.

Experimental

Chemicals and reagents

Lenalidomide raw material (purity 98.5%) was obtained from Reliance Chemical (Pomba, India). HPLC quality deionized water, acetonitrile, methanol, ethanol, formic acid were purchased from Merck (Darmstadt- Germany).

Instrumentation

HPLC system: Dionex ultimate 3000 for instrument equipped with a Diode array Detector DAD-RS 3000 (Dionex-Germany), LC pump (model), auto sampler (model), column oven, and windows 7-Chromeleon 7.2 software chromatography data system. LC-MS, LC: (Agilent 1200), mass detector: API 3200-AB Sciex triple quad, ESI ionization technique and ultra violet lamp (Chromato-Vue-C-70G). The applied analytical method was taken from the publication for Shu *et al* and modified to suit this work [16]. Mobile phase was composed of the formic acid solution (pH 3.0) and acetonitrile (90:10 v/v), respectively, then filtered through 0.45 µm nylon membrane filter and degassed. Inertsil ODS-3V column (25 cm length, 4.6 mm internal diameter, 5 µm particle size) at 25 °C was used as analytical column. The mobile phase was kept at flow rate 1.0 ml/min and the injection volume was 10 µl. Mettler toledo pH meter FP20 and Elma ultrasonic bath S30.

Preparation of Lenalidomide standard solution

10 mg of lenalidomide raw martial was dissolved in 10 ml of acetonitrile to give a concentration of 1.0 mg/ml.

Time-dependent degradation in methanol (or ethanol)

20 mg lenalidomide raw material was mixed with 20 ml methanol (or ethanol) to give a concentration of 1.0 mg/ml, this solution was kept at room temperature in dark place for 10 days. Then, the sample solution was filtrated using 0.45µm Nylon syringe filter.

Heat-dependent degradation in methanol

20 mg lenalidomide raw material was mixed with 2 ml methanol, this solution was then refluxed for 2 days at 70 °C in water bath and diluted with methanol to give a concentration of 1.0 mg/ml. Then, the sample solution was filtrated using 0.45µm Nylon syringe filter.

Results and Discussion

Recently, many published papers examined the chemical stability of lenalidomide starting material towards different stress conditions and by using various HPLC methods but none of them identified the structures of any developed degradants [17–19].

In the present work, a methanolic solution of lenalidomide starting material was prepared at room temperature followed by successive analysis for 10 days using a validated HPLC method [16]. HPLC chromatogram (Fig. 2, A) shows two significant degradants at RRTs 1.3 and 1.8 with a relative intensity of (1:3) on the tenth day. None of these new degradants belongs to any of the known impurities [20].

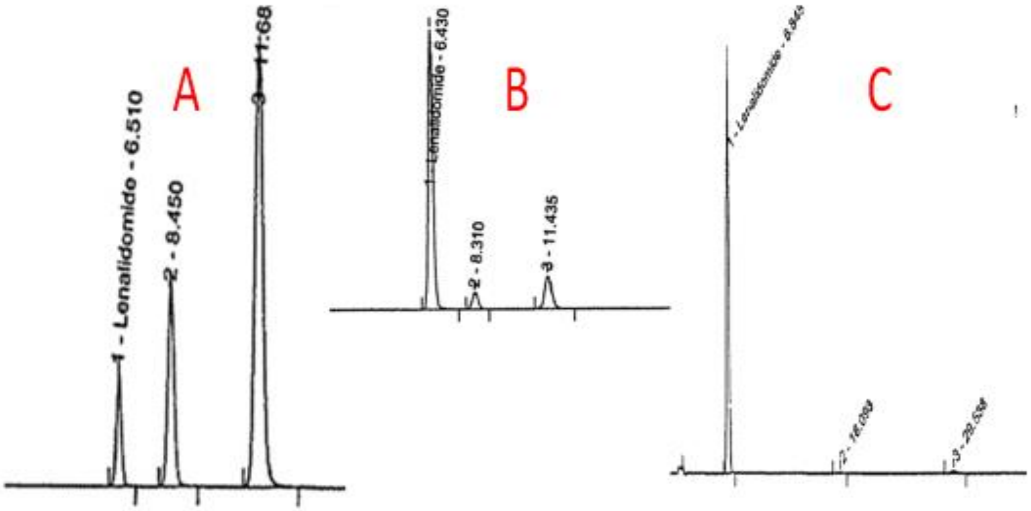


Fig. 2. HPLC Chromatogram of methanolic solution of lenalidomide stored at room temperature for 10 days (A), methanolic solution of lenalidomide refluxed for 2 days (B) and ethanolic solution of lenalidomide stored at room temperature for 10 days (C). The first peak in each chromatogram for lenalidomide.

To investigate the effect of heat on the formation of these corresponding degradants, a methanolic solution of lenalidomide was refluxed for 2 days then analyzed by employment the same analytical method, the corresponding HPLC chromatogram (Fig. 2, B) shows the same two degradants (same RRT's) but depicted lower area percent 6 and 15%, respectively.

Other organic solvents (ethanol, higher alcohols and acetonitrile) were employed to examine the effect of these solvents on the chemical stability of lenalidomide but unfortunately, lenalidomide has a limited solubility in higher alcoholic solvents. However, a suspended solution of lenalidomide in ethanol was prepared and stored at room temperature for 10 days. HPLC chromatogram (Fig. 2, C) shows two degradants at RRT's 2.64 and 4.31 resulted with area percent 0.37 and 1.71%, respectively. It is interesting to

note that the area percent of lenalidomide was 97.91% which may indicate that the response factors of both degradants are almost identical. On the other hand, a solution of lenalidomide in acetonitrile, which was prepared and stored for the same time interval showed no degradants at the specified retention times (after the peak of lenalidomide).

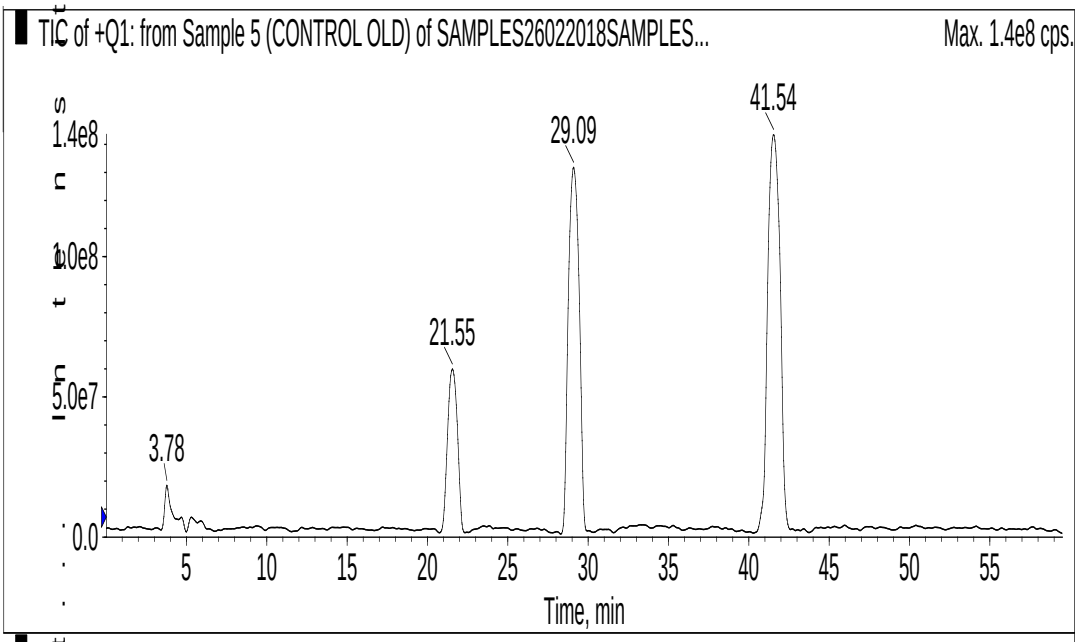
LC-MS analysis was employed to determine the masses of these interesting degradants. The proton adduct of the molecular ion of lenalidomide is 260 m/z, based on the positive mode of analysis. Other relevant lenalidomide peaks have the masses of 282 and 298 which belong to $[M+Na]^+$ and $[M+K]^+$, respectively. It is interesting to note that the molecular masses of both degradants is the same (*m/z* 292), in addition, two extra peaks are also observed at *m/z* 314 and 330 due to the formation of sodium and potassium adducts (Fig. 3). The fragmentation pattern of MS spectra is similar for these two degradants.

From the polarity point of view, lenalidomide and its two degradants are significantly different in this respect. Under the present analytical conditions, lenalidomide has the shortest retention time and therefore considered the most polar one (predicted *log p* -0.83). While, other degradants (RRTs 1.3 and 1.8) have predicted *log P* values -0.14 and 0.02, respectively. Based on the MS and polarity data, chemical structures of these degradants were proposed as shown in Fig. 4 and their names as 4-amino-2-(2-hydroxy-2-methoxy-6-oxopiperidin-3-yl)isoindolin-1-one (**A**) and 4-amino-2-(6-hydroxy-6-methoxy-2-oxopiperidin-3-yl)isoindolin-1-one (**B**).

We supposed that these degradants are formed by the mechanism of nucleophilic addition of methanol to the carbonyl group; other related chemical structures were examined but excluded since none of them is completely compatible with obtained experimental data in terms of molar masses and polarity. For example, ring opening products of the intermediates in Fig. 4 were proposed since their molar masses are *m/z* 292 but they were excluded since their predicted *log P* values were -0.82 for both structures (closer to the *log P* value of lenalidomide) which means that they should have retention times closer to that of lenalidomide. In addition, ring opening reactions cannot be produced unless lenalidomide is hydrolyzed under vigorous experimental conditions since cyclic imide group is relatively stable.

Interestingly, in rhodium catalyzed carbomination of substituted alkene, it has been found that the usage of methanol as a solvent is crucial for improving the efficiency of the reaction relying on the reactivity of enoxyphthalimide [21]. The latter is an imide ring resembles piperidone-2,6-dione part of lenalidomide (Fig.1). Deep insight for the methanol reactivity towards the imide ring was introduced by Chen *et al.* [22], they found that methanol-assisted ring opening of imide group in phthalimide undergoes stepwise mechanism rather than a concerted opening. Their calculations justified the occurrence of a relatively stable tetrahedral intermediate resulted from the attack of imide ring by a methanol molecule before the ring is opened. A structure similar to our proposed degradants in Fig.4 which represents the tetrahedral intermediates of methanol addition to piperidone-2,6-dione part of lenalidomide. According to Chen *et al* calculations, the energy barrier of the concerted ring opening of phthalidimide is quite high whereas the barriers of stepwise mechanism is lesser and more acceptable in justifying the reaction occurrence at room temperature. Moreover, it has been reported that the activation energy of tetrahedral intermediate is almost similar to the activation energy of phthalimide opening from the intermediate [22]. Therefore, these calculations justified the relative stability and occurrence of our suggested degradants of lenalidomide (Fig. 4). Additionally, using methanol as a solvent might participate in stabilizing the tetrahedral intermediates due to excessive hydrogen bonding formation between methanol as a solvent and the methoxy and hydroxyl groups of the degradants.

A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21.55
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15
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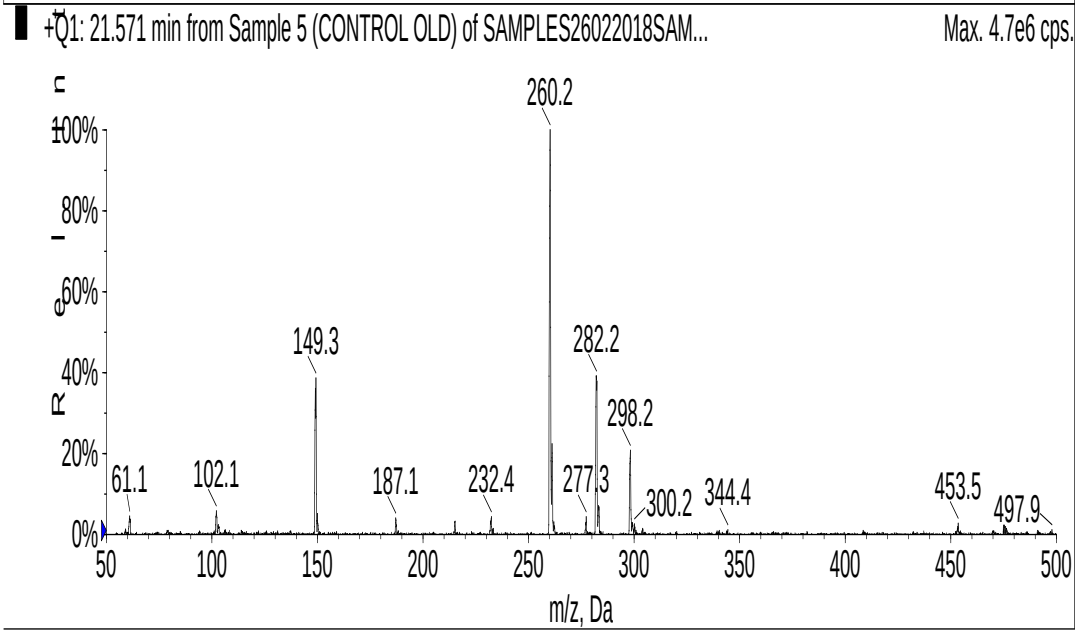
Time, min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100%
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61.1
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149.3
187.1
232.4
260.2
277.3
282.2
298.2
300.2
344.4
453.5
497.9
50
100
150
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250
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350
400
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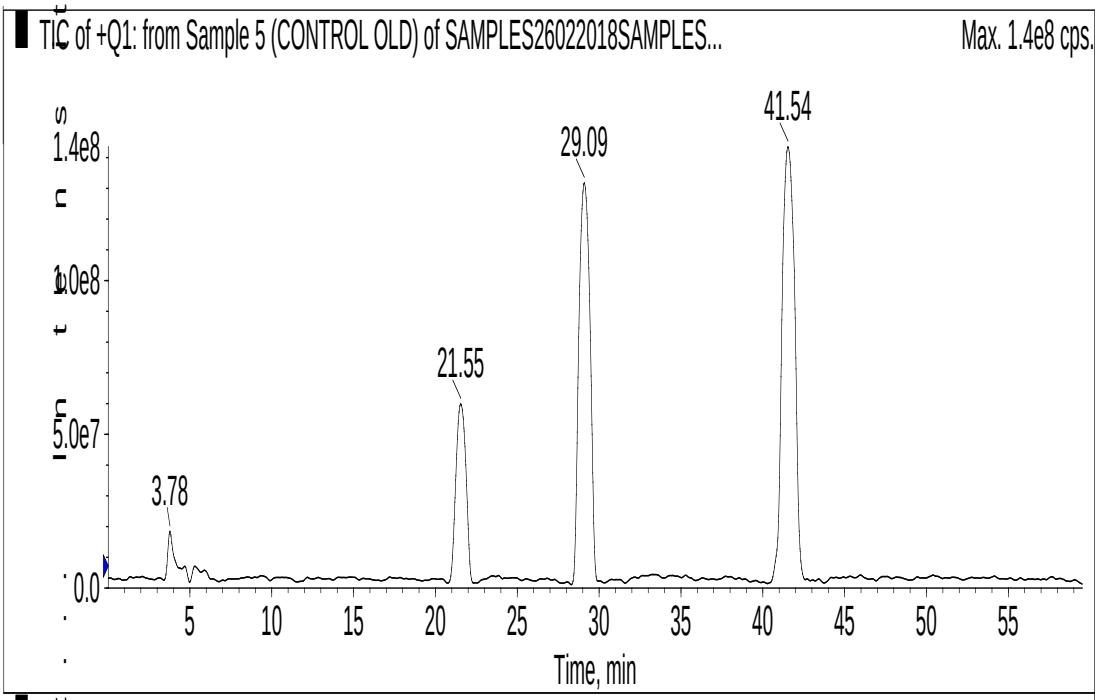
500
m/z, Da

B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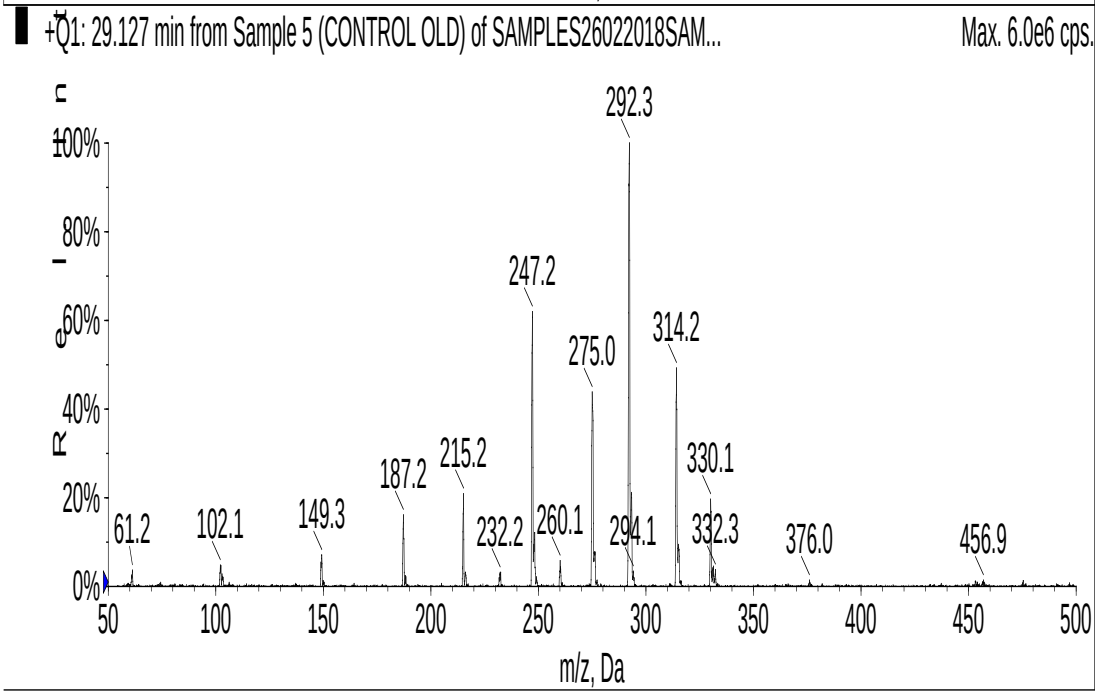
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Max. 6.0e6 cps.
100%
80%
60%
40%
20%
0%
61.2
102.1
149.3
187.2
215.2
232.2
247.2
260.1
275.0
292.3
294.1
314.2
330.1
332.3
376.0
456.9
50
100
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200
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m/z, Da

C

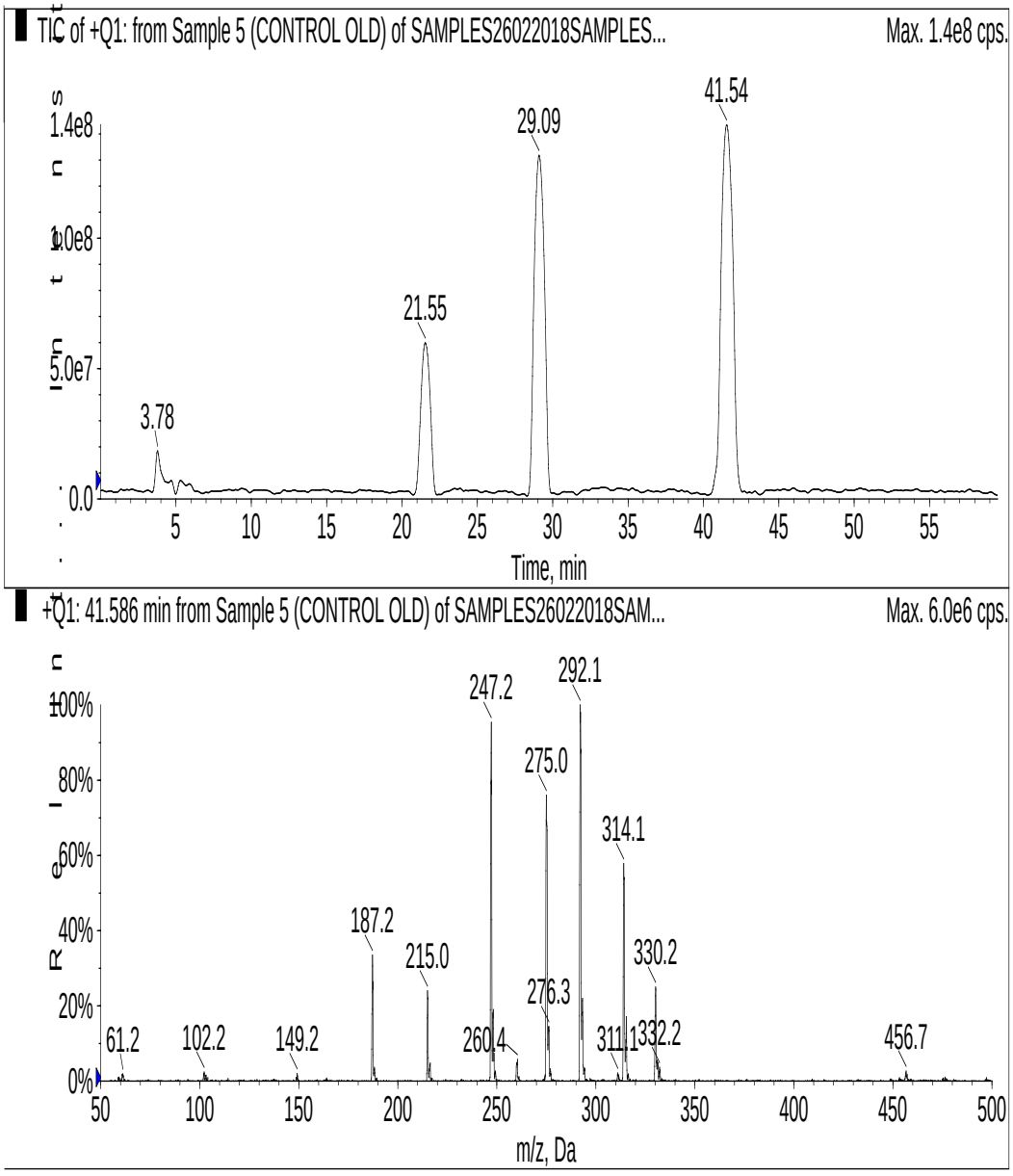

Fig. 3. LC-MS chromatograms and MS charts for degradants at RTs 29.1and 41.5 min. (A) denotes Lenalidomide chromatogram and mass chart.

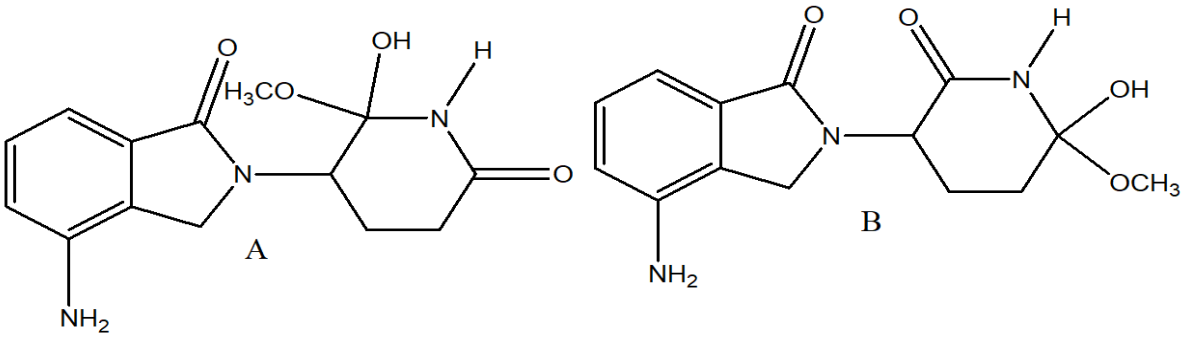

Fig. 4. Proposed chemical structures of the two degradants A and B (RRTs 1.3 and 1.8, respectively).

Conclusions

Two new degradants have been detected when methanolic solution of lenalidomide is prepared and kept at room temperature accomplished with increasing their amounts with time. It is highly advisable to monitor these degradants in various pharmaceutical samples (e.g. stability samples) in case alcoholic solvents, particularly methanol, are employed during the formulation and analysis processes.

Acknowledgements

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List of abbreviations

LC-MS	**Liquid chromatography-mass spectrometry**
API	Active pharmaceutical ingredient
API	Atmospheric pressure ionizer
ESI	Electrospray ionizer
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MDS	Myelodysplastic Syndrome
US-FDA	United states-food and drug administration
ODS	Octadecyl silane
$[M+Na]^+$	Sodium adduct of molecular ion
$[M+K]^+$	Potassium adduct of molecular ion
M / Z	Mass over charge
MS	Mass spectrometry
RRT	Relative retention time

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التحقق من الاستقرار الكيميائي لليناليدوميد في مذيبات الميثانول / الإيثانول باستخدام الطور العكسي للكروماتوغرافيه والكروماتغرافيه السائلة مع مطياف الكتلة

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ملخص

لينااليدوميد دواء غير متجانس الحلقة يستخدم لعلاج متلازمة ميلادوبلاستيك. يركز البحث الحالي على الشكل البنيوي للمواد المتحللة الجديدة التي تتشكل بصوره غير متوقعه عند تخزين اللينااليدوميد في الميثانول، متبوعًا باقتراح آلية تكوينها. الاشكال المقترحة للمواد المتحللة مستقرة نسبيًا حيث ينتج وسيطان رباعي السطوح من إضافة ميثانول محبة للنواة إلى كربون مجموعة الكربونيل في حلقة الإيميد. قد تساهم جزيئات الميثانول، كمذيب، في تثبيت المادة الوسيطة عبر تكوين رابطة الهيدروجين معها. تم العثور على هذه المواد المتحللة بشكل وفير في محلول لينااليدوميد/ ميثانول. لذا فإن التقييم السمي لها أمر يعتبر بالغ الأهمية.

الكلمات الدالة: لينااليدوميد، المواد المتحللة، الطور العكسي للكروماتوغرافيه، مقياس الطيف الكتلي.

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