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Development and Validation of Gas Chromatography Method for the Determination of 2,2'-Azobisisobutyronitrile and Di-tertiary Butyl Dicarbonate Contents in Lenalidomide Drug Substance

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ABSTRACT

A simple capillary gas chromatography method using a flame ionization detector has been developed and validated for the quantitative determination of trace analysis of 2, 2'-Azobis-Iso-Butyro-Nitrile (AIBN) and Ditertiary Butyl dicarbonate (DIBOC) in Lenalidomide [LEN] drug substance. Efficient chromatographic separation was achieved on SPB-1, 30 m long with 0.53 mm; 1.0 µm film thickness consists with 100% Dimethylpolysiloxane as stationary phase. The run time was 25 min, employing programmed temperature with split mode (1:5). The analytical method validation is essential for analytical method development and tested extensively for Specificity, Linearity, Precision (repeatability and reproducibility), Accuracy, Robustness, Range, Limit of Quantization (LOQ) and Limit of Detection (LOD). The achieved Limit of Detection (LOD) values were 3.7, 4.0 µg/g, limit of quantization (LOQ) values were 11.2, 12.0 µg/g for AIBN and DIBOC respectively. The method was found to be linear in the range between 3.7 µg/g and 2250 µg/g with correlation coefficient 0.9999 and 0.9999 for AIBN and DIBOC respectively. The average recovery range obtained for these two impurities was between 100.0% and 101.4%. The developed method was found to be linear, robust and rugged for the determination of AIBN and DIBOC in LEN drug substance. The obtained experimental results are discussed in this present article.

Keywords: 2, 2'-Azobisisobutyronitrile; Di-Tert Butyl Dicarbonate; Lenalidomide; GC-FID; Validation

INTRODUCTION

Lenalidomide (LEN) chemically known as (RS)-3-(4-amino-1-oxo-3H-isoindol-2-yl) piperidine-2,6-dione). The Empirical Formula is $C_{13}H_{13}N_3O_3$ and the Molecular weight is 259.261 g/mol. LEN is classified as a thalidomide analogue, immunomodulatory agent and an anti-angiogenic agent. LEN is used in the treatment of hematological malignancies, particularly multiple myeloma and a Myelodysplastic Syndrome (MDS) patients with deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities and is recommended 10 mg dose for daily, if my cause thrombocytopenia or neutropenia, it may reduce to 5.0 mg every other day. LEN has shown promise in Phase-II studies for chronic lymphocytic leukemia, non-Hodgkin's lymphoma, amyloidosis and myelofibrosis with myeloid metaplasia. It may act by inhibiting the growth of new blood vessels (angiogenesis) in tumors, enhancing the status of the immune system, or decreasing cytokine and growth factor production. The strong evidence-based clinical success of LEN has led to its recent approval by the US Food and Drug Administration (US-FDA) under the trade anme Revlimid® by Clegene Corporation (New Jersey, USA). Lenalidomide has three main activities: direct antitumor effect, inhibition of the microenvironment support for tumor cells, and immunomodulatory role. LEN induces tumor cell apoptosis directly and indirectly by inhibition of bone narrow stromal cell support, by anti-angiogenic and anti-osteoclastogenic effects, and by immune-modulatory activity. LEN has a broad range of activities that can be exploited to treat many hematologic and solid cancers (Figure 1) [1].

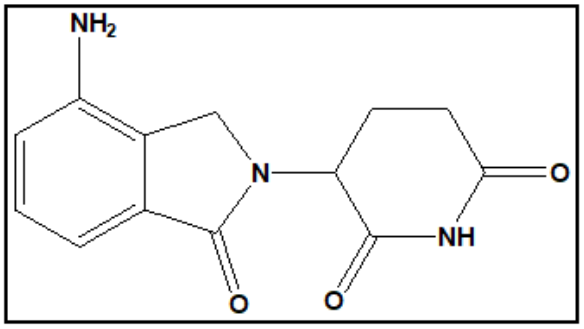

Figure 1: Chemical Structure of Lenalidomide.

In the process of Lenalidamide, Methyl-2-methyl-3-nitro benzoate is the key raw material. In the first step of the preparation of Lenalidamide, Bromination of Methyl-2-methyl-3-nitro benzoate with N-Bromosucinamide in presence of 2,2'-Azobis-Iso-Butyl-Nitrile (AIBN) (Used as catalyst) and Methanol to convert the Methyl-2-bromomethyl-3-nitro benzoate. In next stage, Methyl-2-bromomethyl-3-nitro benzoate added with 3-Amino-piperidine-2,6-dione hydrochloride to form Lenalidomide nitro precursor. Finally this nitro compound undergo reduction to convert Lenalidomide. In the preparation of 3-Amino-piperidine-2,6-dione hydrochloride, L-Glutamine and Di-Tert Butyl-Di-Carbonate (DIBOC) used as starting materials [2]. Azo-Iso-Butyro-Nitrile (AIBN) and Di-Tetra Butyl-Di-Carbonate (DIBOC) Chemicals are used in the manufacturing process of Lenalidimide and these are not listed in ICH or other Genotoxic guidelines (Figures 2 and 3).

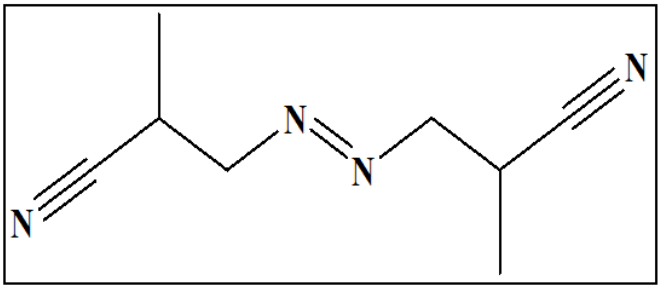

Figure 2: Chemical Structure of Azo-Iso-Butyro-Nitrile (AIBN).

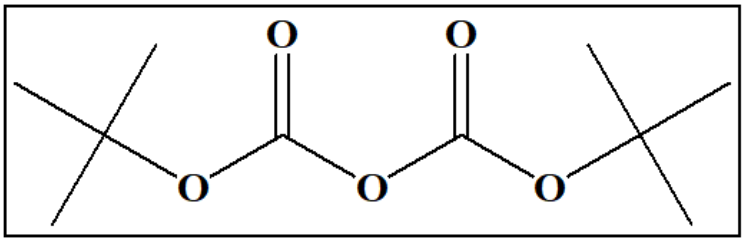

Figure 3: Chemical Structure of Di-Tetra Butyl-Di-Carbonate (DIBOC).

Impurity other than active drug substance is to be controlled with appropriate limit in the drug substance irrespective of harmful nature as per International Conference of Harmonization (ICH) guidelines on impurities. Consequently these impurities may be retained or may not be retained in final stage of drug substance. These impurities quantification were required in final drug substance. To the best of our knowledge no report has been published on the analysis of AIBN and DIBOC in Lenolidamide drug substance in literature. Subsequently, Gas Chromatography (GC) method was optimized to determine the contents of AIBN and DIBOC in Lenolidamide drug substance with specification level for each of component is 0.15% (1500 µg/g) [3].

MATERIALS AND METHODS

Solvents, Chemicals and Samples

2, 2'-Azo-Bis-Iso-Butyro-Nitrile (AIBN), Di-tert butyldicarbonate (DIBOC), Dodecane, Methanol, n-Hexane, Isopropyl alcohol, Tetrahydrofuran, Triethylamine, toluene, Ethyl acetate, t-Butanol, Acetic acid and N,N-dimethylformamide were procured from Sigma Aldrich (Steinheim, Germany). Methylene chloride (Analytical grade, use as Diluent), Sodium Hydroxide pellets, HPLC water were procured from MERCK chemicals (Mumbai, India). The investigated drug substance LEN was gifted from NATCO Research Centre Laboratories. [4].

Instrumentation: A gas chromatograph 7890 A equipped with flame ionization detector with G1888 auto sampler (Make: Agilent Technologies, Santa Clora, CA, USA) and with data acquisition and processing using Empower 3 Software Build 3471 were used in research work [5].

Chromatographic conditions and methodology: The chromatography separation was carried out on SPB^{-1} capillary column with fused silica coated with 100% Dimethylpolysiloxane stationary phase, Make: Supelco with a dimension of 30 m length, 0.53 mm I.D. film thickness 3.0 µm. Helium is used as carrier gas for entire experiments as it is provides good base line and well resolution between AIBN, DIBOC and Dodecane peaks. The other method parameters, which were used for this work has given below:

- **Injector temperature:** 225°C
- **Detector temperature :** 275°C
- **Detector:** Flame Ionization Detector (FID)
- **Carrier gas:** Helium
- **Carrier gas flow:** 5.0 ml/min
- **Spit ratio:** 1:5
- **Run time:** 25 min
- **Injection volume:** 2 µL, 10°C/min.

Preparation of Solutions

1N sodium hydroxide solution: Accurately weigh and transfer 4.0 g of Sodium hydroxide pellets into a 100 mL, clean, dry volumetric flask containing about 50 mL water, dissolve and make up to dilute to volume with water [6].

Internal Standard solution (IS): Accurately weigh and transfer 93 mg of Dodecane into a 25 mL clean, dry volumetric flask containing about 15 mL of Methylene chloride, mix and make up to volume with Methylene chloride. Dilute 5.0 mL of this solution to 250 mL with Methylene chloride [7].

Blank solution: Into a clean, dry glass centrifuge tube, take 2 mL of 1N NaOH solution and add 3 mL of internal standard solution and shake vigorously for 1 min. Allow the two phases to separate. Collect the lower layer (Methylene chloride layer) and transfer into GC vial and inject into GC [8].

Standard solution: Accurately weigh and transfer 37.5 mg of AIBN and 37.5 mg of DIBOC into a 25 mL clean, dry volumetric flask containing about 10 mL of internal standard solution, dissolve and make up to volume with internal standard solution. Dilute 5.0 mL of this solution to 50 mL with internal standard solution. Into a clean, dry glass centrifuge tube, take 2 mL of 1N NaOH solution and add 3 mL of standard solution and shake vigorously for 1min. Allow the two phases to separate. Collect the lower layer (Methylene chloride layer) and transfer into GC vial and inject into GC [9].

Sample solution: Accurately weigh and transfer 300 mg of LEN sample into a clean, dry glass centrifuge tube, add 2 mL of 1N NaOH solution and shake to dissolve the sample. Add 3 mL of internal standard solution and shake vigorously for 1 min. Allow the two phases to separate. Collect the lower layer (Methylene chloride layer) and transfer into GC vial and inject into GC [10].

RESULTS

Method Development and Optimization

The objective of this work is to determine low level concentration of AIBN and DIBOC in LEN drug substance by using GC-FID which is easily available instrument, good separation and desired sensitivity. No analytical methods available in literature to quantifying AIBN and DIBOC by GC-FID. The present investigation was initiated for the quantification of AIBN and DIBOC by GC-FID technique in Lenalidomide drug substance, as GC instrument is most properly available at all laboratory locations and easy to handle in all existing method conditions [11].

There was no possibility for UV or Fluorescence detection to quantifiy AIBN and DIBOC, as no chromopore present in this analytes. Hence, gas chromatography was selected. Further, the method development trails carried out based on LEN, AIBN and DIBOC solubilities. Initially, DB-624, 30 mm long with 0.53 mm ID, 3.0 µm Particle diameter columns consisted with 6% Cyanopropylphenyl-94%-dimethylpolysiloxane as stationary phase and direct injection technique with Dimethylsulfoxide has been selected with following temperature programme at constant Flow 5.0 ml/min [12].

Column oven temperature programme: Further, standard solution has been prepared with respect to sample concentration based on 1500 µg/g limit as discussed in introduction section. By using all the above method parameters and solutions, we have started work. In this trail, peak shapes were not good, interference from sample matrix and low response of analyte has been observed. During method optimization we have tried with more solvents to avoid interference and response issues. But not achieved by direct injection technique. Hence, the extraction procedure with Sodium Hydroxide and Methylene chloride and internal standard Dodecane have been selected as mention in chromatographic conditions and methodology section. In this trail, sample interference issue has resolved but analyte peak shape and response was good. Finally, column has changed to SPB-1, 30 m, 0.53 mm, 3.0 µm and programe has changed with constant Flow 5.0 ml/min.

$$80^{\circ}C\ (3\ min) \longrightarrow 250^{\circ}C\ (7\ min)$$

Column oven temperature programme: Finally satisfactory separation with better peak shapes and response were achieved on chromatographic conditions. The optimized chromatographic conditions and sample preparations already discussed in earlier sections [13].

$$100^{\circ}C\ (3\ min) \longrightarrow 10^{\circ}C/min\ 250^{\circ}C\ (7min)$$

Method validation: The optimized GC method was validated according to ICH guideline Q2 (R1). The method was validated for linearity and range of 3.7 µg/g to 2250 µg/g. Accuracy, Precision (System precision, Method precision and intermediate precision), Specificity, Limit of Detection (LOD), Limit of Quantification (LOQ) and Robustness. All validation experimental results met the pre-established acceptance criteria and demonstrated the method was suitable for its intended purpose [14].

DISCUSSION

Specificity

As per ICH, specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed method was verified in presence of residual solvents like Methanol, Methylene chloride, n-Hexane, Isopropyl alcohol, Tetrahydrofuran, Triethylamine, Toluene, Ethyl acetate, t-Butanol, Acetic acid and N,N-Dimethylformamide which are used in the synthesis process of LEN drug substance [15].

For determination of specificity experiment, blank solution, all residual solvents which are used in synthetic process of LEN (Methanol, Methylene chloride, n-Hexane, Isopropyl alcohol, Tetrahydrofuran, Triethylamine, Toluene, Ethyl acetate, t-Butanol, Acetic acid and N,N-Dimethylformamide etc.) including AIBN, DIBOC and Dodecane solutions were prepared individually and injected into GC to confirm the retention times. After that, solution of LEN (control sample), LEN spiked with at 0.15% (1500 ppm) of AIBN and DIBOC (spiked sample) and LEN spiked with all residual solvents including 0.15% (1500 ppm) of AIBN and DIBOC (all spiked sample) solution, were prepared as per given methodology and injected into GC at the given chromatographic conditions. Based on obtained data, AIBN and DIBOC peaks are well separated from all other solvents and indicating that the test method is selective and specific for the determination of AIBN and DIBOC in LEN. Retention times of all solvents (Table 1) and spiked sample data (Table 2). Typical GC chromatograms of Blank solution, Standard solution, Control sample (As such sample), Spiked sample and All spiked sample solutions (Figures 4-8).

Table 1: Individual injections of all residual solvents.

Solvent name	RT(min)
AIBN	7.399
DIBOC	10.596
Dodecane	10.901
Methanol	1.564
Isopropyl alcohol	1.738
Dichloromethane	1.769
Ethyl acetate	2.199
Tetrahydrofuran	2.374
Toluene	3.803
Triethylamine	2.841
t-Butanol	2.153
Acetic acid	2.024
n-Hexane	2.235
N,N-Dimethylformamide	3.557

Table 2: All spiked sample (Lenalidomide drug substance spiked with AIBN and DIBOC including all residual solvents)

Solvent name	RT(min)	RRT	Resolution
Methanol	1.569	0.14	-
Isopropyl alcohol	1.738	0.16	6.79
Methylene chloride	1.769	0.16	0.34
t-Butanol + Ethyl acetate	2.199	0.2	3.76
Tetrahydrofuran	2.374	0.22	2.15
Triethylamine	2.847	0.26	5.71
N,N-Dimethylformamide	3.527	0.32	8.53
Toluene	3.804	0.35	3.55
AIBN	7.374	0.68	41.06
DIBOC	10.564	0.97	35.07
Dodecane	10.87	1	3.68

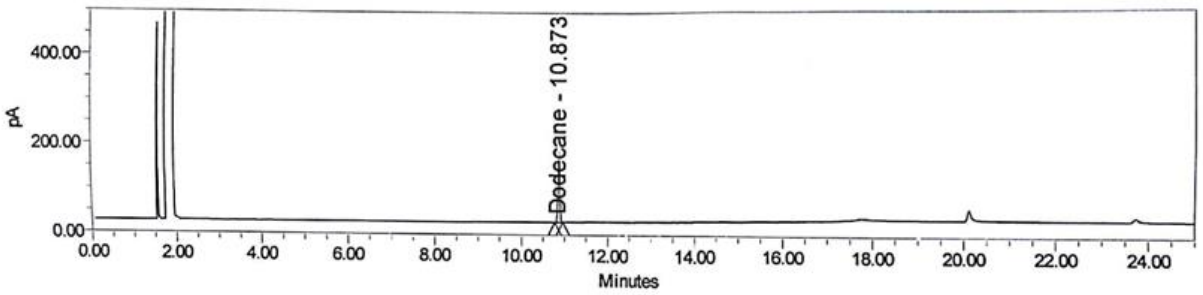

Figure 4: Blank solution of typical GC chromatograms.

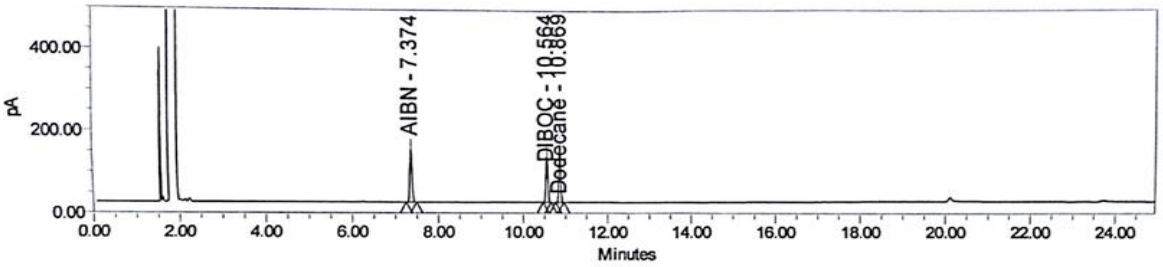

Figure 5: Standard solution of typical GC chromatograms.

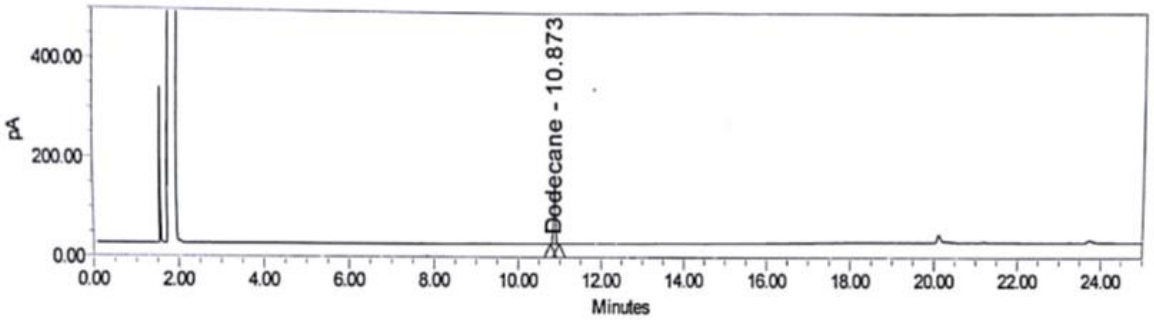

Figure 6: Lenalidomide drug substance (as such sample) of typical GC chromatograms.

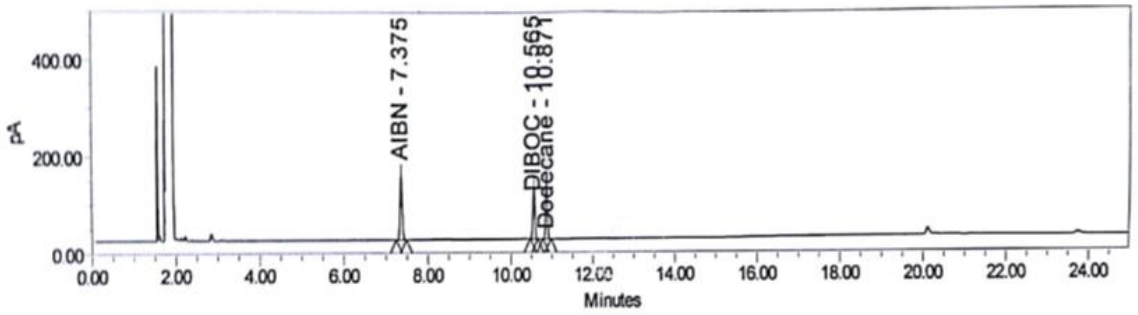

Figure 7: Lenalidomide drug substance spiked with AIBN and DIBOC of typical GC chromatograms.

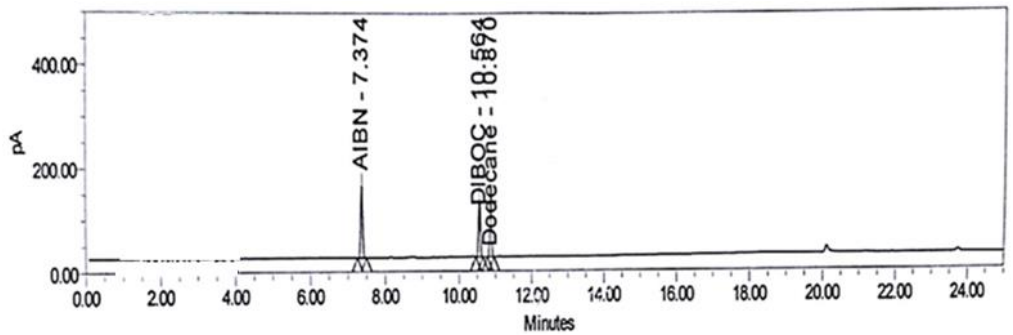

Figure 8: Lenalidomide drug substance spiked with AIBN and DIBOC and all residual solvents of typical GC chromatograms.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Standard solution (0.15% concentration) of AIBN and DIBOC were prepared and injected into gas chromatograph. The Limit of Detection (LOD) and Limit of Quantification (LOQ) values for AIBN and DIBOC were determined by Signal to Noise ratio (S/N) method. The minimum concentration at 3:1 S/N was considered as LOD and the concentration at 10:1 S/N was established as LOQ. The predicted LOD and LOQ values obtained for AIBN and DIBOC were 3.7 µg/g, 11.2 µg/g, 4.0 µg/g and 12.0 µg/g respectively for both with respect to sample concentration. Precision was verified by preparing the solutions at about LOD and LOQ concentrations and injected each solution six times in to GC and the achieved précised values are given in Table 3. GC chromatograms of LOD solution and LOQ solutions (Figures 9 and 10) [15].

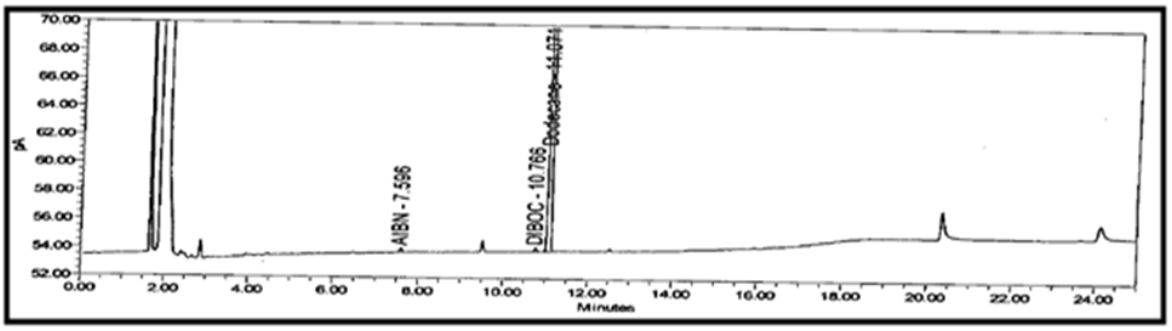

Figure 9: Typical GC chromatograms of LOD solution.

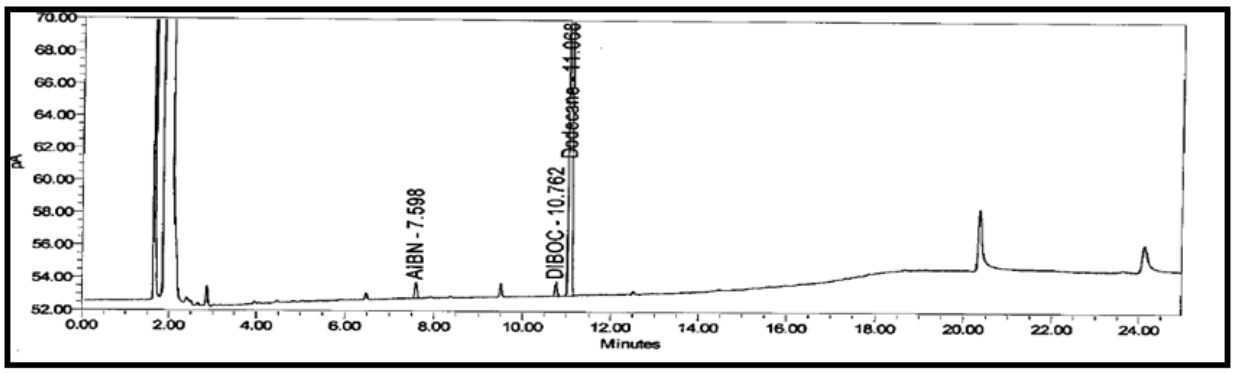

Figure 10: Typical GC chromatograms of LOQ solution.

Linearity: The linearity was evaluated by measuring area ratio for AIBN and DIBOC with respective internal standard (Dodecane) over concentration range of 3.7 µg/g to 2250 µg/g (LOQ to 150% of specification level) with respect to sample concentration for both AIBN and DIBOC. The obtained data was subjected to statistical analysis using a linear regression model. The statistical values like Slope, Intercept, STEYX and Correlation coefficient from linearity plot drawn for concentration versus area ratio of AIBN/Internal standard and DIBOC/Internal standard. Duplicate injections were performed at each level (Table 3) [16].

Table 3: Statistical data of LOD, LOQ and linearity experiment.

Statistical parameters	Experimental results	
	AIBN	**DIBOC**
LOD and LOQ experiment		
Limit of Detection (LOD) (µg/g)	3.7	4
Limit of Quantification (LOQ) (µg/g)	11.2	12
Precision for LOD (RSD%) (n=6)	3.7	15.4
Precision for LOQ (RSD%) (n=6)	1.2	5.4
Linearity experiment		
Correlation coefficient	0.9999	0.9999
Concentration range (µg/g)	11.2-2108	12.1-2266
Intercept	0.0026	-0.0094
Slope	0.0008	0.0006
STEYX	0.0091	0.0094
No. of points covered	8	8

Accuracy: Accuracy experiment was performed using standard addition technique. The recoveries were determined by spiking known amount of AIBN and DIBOC at four levels i.e. LOQ level, 50%, 100% and 150% of specification level i.e.1500 µg/g into LEN. These samples were prepared and analyzed in triplicate. The calculated recovery values AIBN ranged from 93.8% to 100.9%, for DIBOC ranged from 87.5% to 102.3%. Average recovery of four levels (twelve determinations) was 98.6% and 98.9% for AIBN and DIBOC respectively. **N**itriles are the building blocks of most biologically active substances and natural products. However, amides and carboxylic acids have synthesized from nitriles, due to this α-aminonitriles have occupied great position in synthetic organic chemistry, this growing demand of nitriles, satisfied by Strecker reaction. Strecker in 1850 reported the synthesis of α-aminonitriles by multicomponent condensation of aldehyde, amine and hydrogen cyanide hydrocyanation of emines is thus basic C-C bond formaon reaction involves conversion of nitriles to carbonyl group. Modified Strecker reaction i.e. synthesis of optically active α-amino acid by the hydration of cyanide, α-aminonitriles is acts a precursor fragment for the synthesis of α-amino acid, imidazole and several biologically active compounds containing nitrogen atom. Bifunctionality of α-aminonitriles acts as a building blocks of pharmaceutical industries, such as serine protease inhibitors, (-, +) phtalascidine 650 and also in the synthesis of boron containing retinoids. Synthesis of heterocyclic moiety such as 1,2,3-diazaphospholidines, imidazole, oxazoles, and isothiozoles derived from 2-amino-2-alkyl (aryl) propanenitriles as a starting material. Synthesis of 5-amino-4H-imidazoles was achieved by reacting α-aminonitriles with imidoester which is a key material of many biological compounds. Different protocols have been reported for the synthesis of α-aminonitriles such as Formic acid, ammonium chloride, PPh_3/DEAD, Bicyclic Guanidine, polyethylene glycol (PEG-OSO_3H), MgI_2, sulphated polyborate, PEG -400, Zn (CN), cinchona-based thiourea alkaloid, 5mol % to 20mol % L-prolineamide derived N,N'-dioxide, $Ga(OTF)_3$, Nafion-H and NafionSAC-13, SBA-15 supported sulphonic acid, indium (III) iodide, mesoporous MCM-41 catalyst, ionic liquid [bmim] BF_4 or $MgBr_2.OEt_2$, Bismuth Nitrate, $Fe_3O_4SiO_2$MeandEt-$PhSO_3H$, task-specific ionic liquid, chiral ammonium trifluoroacetate, potassium hexacyanoferrate (II), Silica based Scandium (III), Pd (II), magnetically separable nanoparticles. We have reported here environmentally green EPZG as catalyst for the synthesis of α-aminonitriles. EPZG is a $FeCl_3$ supported on clay (Tables 4 and 5) [17].

Table 4: Accuracy data of AIBN.

Accuracy	Level-I	Level-II	Level-III	Level-IV
(Average of 3 replicates)	**(at LOQ)**	**(at 50%)**	**(at 100%)**	**(at 150%)**
Added(µg/g)	11.2	702	1404	2106
Found(µg/g)	10.5	706	1411	2085
Recovery (%)	94.3	100.6	100.5	99
RSD (%)	0.5	0.2	0.1	0.1
Overall recovery (%)			98.6	
(Average of 12 replicates)				

Table 5: Accuracy data of DIBOC.

Accuracy	Level-I	Level-II	Level-III	Level-IV
(Average of 3 replicates)	**(at LOQ)**	**(at 50%)**	**(at 100%)**	**(at 150%)**
Added(µg/g)	12	755	1509	2264
Found(µg/g)	11	765	1537	2283
Recovery (%)	91.7	101.4	101.8	100.9
RSD (%)	4.2	0.2	0.6	0.1
Overall recovery (%)			98.9	
(Average of 12 replicates)				

Precision: The precision was the study of the method using repeatability and reproducibility (Ruggedness). The performance of the method was evaluated with replicate injections of standard and sample solutions. Standard solution was analyzed by injecting six times for checking the performance of the gas chromatograph system under the chromatographic conditions on the day tested (system precision).The system precision was demonstrated by injecting standard solution of AIBN and DIBOC six times into gas chromatograph system and calculated the area ratios obtained from AIBN/Dodecane and DIBOC/Dodecane.

The relative standard deviation for AIBN and DIBOC 0.1% and 0.1% respectively. Repeatability and reproducibility of the method was studied by analyzing six sample solutions separately. Repeatability was the intra-day variation (method precision) demonstrated by preparing six sample solutions individually using a single batch of LEN drug substance spiked with AIBN and DIBOC at a known concentration level about 1500 µg/g as per methodology and content was determined. The relative standard deviation for the content of AIBN and DIBOC is 0.1% and 0.6% respectively. The intermediate precision was the inter-day variation (ruggedness) was defined as the degree of reproducibility obtained by following the same procedure as mentioned for method precision experiment. Ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in method precision experiment) by spiking AIBN and DIBOC to LEN and injected into different column, different instrument and different analyst on different days (Tables 6 and 7).

Table 6: Statistical data of precision experiments of AIBN.

Injection ID	System Precision	Method Precision	Ruggedness
	Ratios of area counts	AIBN content, µg/g	AIBN content, µg/g
	[AIBN/Dodecane]		
1	1.1354	1412	1568
2	1.1373	1410	1586
3	1.1378	1411	1582
4	1.1351	1411	1567
5	1.1375	1406	1562
6	1.1351	1409	1570
Mean	1.1364	1410	1573
SD	0.0013	2.1	9.4
RSD (%)	0.1	0.1	0.6
95% Cl (±)	0.0014	2	10
Overall statistical data (n=12)	Mean	1491	-
	SD	85.2	-
	RSD (%)	5.7	-
	95% Cl (±)	54	-

Robustness: This study was performed by making deliberate variations in the method parameters. The study was carried out with respect to flow/pressure variation of carrier gas initial pressure and ramp temperature ± 10% and column oven initial temperature and ramp temperature ± 2°C as follow.

Table 7: Statistical data of precision experiments of DIBOC.

Injection ID	System precision	Method precision	Ruggedness
	Ratios of area counts	DIBOC content, µg/g	DIBOC content, µg/g
[DIBOC/Dodecane]			
1	0.9515	1541	1581
2	0.9542	1525	1603
3	0.9528	1544	1583
4	0.9514	1523	1567
5	0.9539	1532	1553
6	0.9512	1538	1565
Mean	0.9525	1534	1575
SD	0.0013	8.6	17.5
RSD (%)	0.1	0.6	1.1
95% Cl (±)	0.0014	9	18
Overall statistical data (n=12)	Mean	1555	-
	SD	25.5	-
	RSD (%)	1.6	-
	95% Cl (±)	16	-

Conditions: In each robustness conditions remaining gas chromatography conditions are same as per test method.

- Column flow/Pressure (-10%): 4.5 ml/min.
- Column flow/Pressure (+10%): 5.5 ml/min.
- Column oven temperature (-2°C)

Column oven temperature (-2°C): 98°C (3 min) $\xrightarrow{8^\circ\ C/min}$ 250°C (7min).

12° C/min

Column oven temperature (+2°C): 102°C (3 min) ⟶ 250°C (7min).

Test method conditions: Column flow/Pressure: 5.0 ml/min

Column flow/Pressure: 5.0 ml/min ⟶ 250°C (7min).

In each robustness condition, solutions of Blank, Standard and LEN spiked with AIBN and DIBOC at about 1500 µg/g concentration level were prepared per methodology and injected in to GC to confirm the retention times. There is no much variation in the Relative Retention Time (RRT) of AIBN and DIBOC of different deliberately varied robustness conditions from the developed methodology. Hence the test method is robust for all varied conditions. All experiments system suitability results (resolution between DIBOC and Dodecane) (Table 8).

Table 8: Robustness data of AIBN and DIBOC.

Robustness condition	Variation	AIBN		DIBOC		Dodecane		Resolution
		RT, min	RRT	RT, min	RRT	RT, min	RRT	
Methodology (As per test method)	-	7.452	0.68	10.664	0.97	10.972	1	3.69
Flow pressure variation-initial pressure	-10%	7.829	0.76	11.023	0.76	11.332	1	3.74
	10%	7.129	0.71	10.357	0.71	10.665	1	3.69
Temperature variation-initial oven and ramps	-2°C and -2°C/min	8.027	0.74	11.88	0.74	12.255	1	3.96
	+2°C and +2°C/min	6.998	0.73	9.771	0.73	10.034	1	3.53

Solution stability: The standard solution and sample solution were prepared by spiking AIBN and DIBOC at known concentration level to LEN drug substance and stability of the solution was tested as freshly prepared and at different intervals with the gap of every one hour and up to 24 hrs at ambient conditions. The stability of solution was determined by comparing results with freshly prepared standard and sample solutions. The results indicating that standard and sample solution was stable for 24 hrs at ambient conditions.

CONCLUSION

The method validation data demonstrated that the Developed GC method is more sensitive and also specificity of the method was established on GC as well as accurate for the determination of AIBN and DIBOC in Lenalidomide drug substance. Hence the validated GC method can be employed in to the routine analysis for the quantification of AIBN and DIBOC in Lenalidomide drug substance.

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