Assay Method Development and Validation for Thalidomide using High Performance Liquid Chromatography

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Abstract: *A simple, accurate, precise, stability indicating HPLC method was carried for the determination of thalidomide along with its impurities. An HPLC isocratic separation was achieved on Develosil ODS UG-5 column (150mm *4.6*mm*5µm particle size) with a mobile phase 0.01M potassium dihydrogen orthophosphate and Acetonitrile in the ratio of 80:20 (v/v) 1.02gm in 500 ml. The flow rate was set at 0.700ml with a detection wavelength of 297nm. The linearity range of thalidomide and its impurities were 0.99914, 0.99966 and 0.99968 respectively. The drug undergoes degradation under acid, base, H2O2, thermal and humidity conditions. The method was validated for precision, accuracy, ruggedness and robustness as per ICH guidelines.*

Keywords: *Thalidomide, HPLC, Stability indicating, Method validation.*

1. INTRODUCTION:

Thalidomide is chemically known as 2-(2, 6-dioxopiperidin-3-yl)-1*H*-isoindole-1, 3(2*H*)-dione. The molecular formula is $C_{13}H_{10}N_2O_4$. The molecular weight is 258.23 g/mol. Thalidomide is used for a number of conditions including erythema nodosum leprosum, multiple myeloma (in combination with dexamethasone), and various other cancers, for some symptoms of HIV/AIDS, Crohn's disease, sarcoidosis, graft-versus-host disease, rheumatoid arthritis and a number of skin conditions that have not responded to usual treatment. [1]The bacterium that causes tuberculosis is related to leprosy. Thalidomide may be helpful in some cases where standard TB drugs and are not sufficient to resolve severe inflammation in the brain [2, 3]. A few analytical methods were reported for the determination of Thalidomide by HPLC in plasma and in blood from pharmaceutical preparation [4, 5]. Other different methods have been reported for its estimation including HPLC in API [6, 7, 8, 9, 10]. Hence no method has been reported for the determination of thalidomide along with its impurities. The aim of the work was to develop a simple, accurate and cost effective HPLC method for the determination of Thalidomide and its impurities. This method was developed and validated as per ICH guidelines [11].

2. MATERIALS AND METHODS

Chemicals and reagents:

The Samples of Thalidomide and its impurities were obtained from Fortunee Laboratories (P) Ltd, Kakinada, and Andhra Pradesh, India. All other analytical reagents such as Ammonium formate, acetonitrile, potassium dihydrogen orthophosphate, hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%) were obtained from Merck specialty chemicals, Mumbai, India. Milli Q' water is used for the preparation of Solutions. Figure 1 expressed the structure of thalidomide and its impurities.

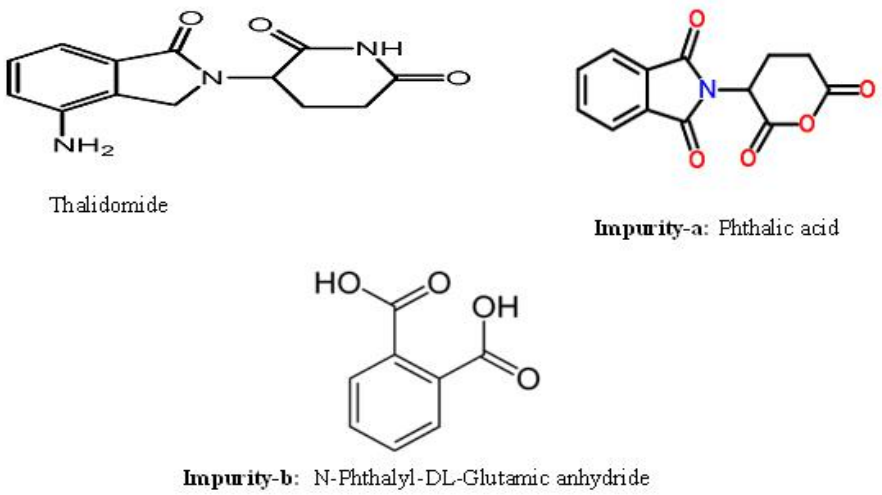

Figure 1:- Structure of thalidomide and its impurities

Instrument specification:

This research has been performed on Agilent make HPLC 1100 instrument. It has binary gradient pump, photo diode array detector (UV), column oven with range of 25°C to 60°C with auto injector. The modules are G1310A isocratic pump with solvent cabinet; G1314A variable wavelength detector (VWD) with standard flow cell (10 mm path length, 14 µl volume, 40 bar maximum pressure) and G2220AA 2D-Value Solution Chem Station.

3. METHODS:

Chromatographic specificication:

The instrument works with a mobile phase of potassium dihydrogen orthophosphate and Acetonitrile in the ratio of 80:20 (v/v) and a column used Develosil ODS UG-5 column (150mm *4.6*mm*5um particle size).The flow rate was maintained at 0.700ml with a detecting wavelength of 297nm.

Impurities standard Stock Solutions:

Weigh accurately about 25 mg of each impurity standards of N-acetyl-Glucosamine, 5- Hydroxymethyl-2-Furaldehyde, Pyrazine, 2- Furaldehyde and Pyrrole-2-Carboxaldehyde then transfer into individual each 50 ml volumetric flasks, add 35 ml of diluent into each flasks, sonicate for 10 minutes to dissolve the material and make up the volume up to mark with diluent and mix.

Diluted Standard solution (Resolution solution):

From the impurities standard stock solution, transfer each 5 ml of solution into 100 ml volumetric flask and make up the volume up to mark with diluent and mix well.

Sample Preparation:

Weigh accurately not less than 20 tablets note down the weight. Then calculate average weight. Crush the 20 tablets in to fine powder with mortar pestle, then transfer an accurately weighed quantity equivalent to 500 mg of Glucosamine Hydrochloride into a 100 ml Volumetric flask, add 70 ml of diluent, sonicate for 30 minutes with intermediate shaking by maintaining sonicator bath temperature below at 28°C. Cool it, make up the volume up to the mark with diluent and mix well.

Centrifuge the Test solution by closing the centrifuge tube tightly with stopper or Para film (to avoid solvent evaporation) at 3000 RPM for 10 minutes, use supernatant solution for filtration. Filter the supernatant solution through 0.45 µm PVDF filter. Inject the clear filtrate solution into HPLC.

Procedure:

Separately inject equal volumes of (about 10 µl) of one injection of diluent as blank, five replicate injections of diluted Standard solution, and one injection of Sample solution into the chromatograph, and record the chromatograms and measure the peak responses. Developed method were validated by following parameters linearity, accuracy, precision, LOD, LOQ and stability testing.

5. RESULTS AND DISCUSSION:

METHOD DEVELOPMENT

System suitability: Standard solution was prepared as per test procedure and injected into the HPLC system as per test method. Evaluated the system suitability parameters and Summarized in the table 1 given below.

Table-1: System suitability results

System suitability parameters		Observed value	Acceptance criteria
Theoretical Plates	Thalidomide	5758	Should be NLT 2000
	Impurity-A	6154	
	Impurity-B	5199	
%RSD	Thalidomide	0.1	Should be NMT 5.0
	Impurity-A	0.6	
	Impurity-B	0.1	
Tailing factor	Thalidomide	1.2	Should be NMT 2.0
	Impurity-A	1.1	
	Impurity-B	1.1	

METHOD VALIDATION

The described method has been validated for thalidomide and its impurities using the following parameters

Linearity:

Linearity was established by plotting a graph between concentration versus peak area and the correlation coefficient was determined. A series of solutions of thalidomide impurities with concentrations ranging from LOQ% to 200% of the target concentration (0.5%) prepared and injected into the HPLC system. The results were given in table 2

Table 2: Linearity data

Parameter	Thalidomide	Impurity a	Impurity b
Linearity range µg/ml	0.03-4.29	0.031-4.1	0.03-4.31
Correlation coefficient	0.99922	0.99924	0.99913
Limit of detection µg/ml	0.0001	0.0002	0.0003
Limit of quantification µg/ml	0.0005	0.0005	0.0012

Limit of Quantitation and Limit of Detection:

A study to establish the Limit of Detection and Limit of Quantification of Thalidomide impurities was conducted.

Limit of Detection and Limit of Quantitation were established based on signal to noise ratio. A series of injections of blank solution were injected and average noise was calculated. Limit of Detection for each impurity was established by identifying the concentration which gives signal to noise ratio about 3. Limit of Quantitation was established by identifying the concentration which gives signal to noise ratio about 10.

Precision:

Stock solutions of impurities were prepared. Six replicates of the drug product were spiked with impurity stock solutions, such that each preparation consists of each known impurity at its specification level (0.2%). Sample solutions were analyzed as per method. The amount of each known impurity in percent in each replicate, the average, and its %RSD were calculated.

Accuracy:

A study of accuracy of thalidomide impurities from spiked samples of test preparation was conducted. Samples were prepared in triplicate at each level by spiking test preparation with LOQ, 50%, 80%, 100%, 150% and 200% of target concentration of thalidomide impurities.The individual values, the % recovery, the % relative standard deviation for % recovery of samples at each concentration level are reported.The results were tabulated in table 3

Table 3: Accuracy for TM

	TM	Impurity A	Impurity B	TM		Impurity A		Impurity B	
Samples	Amount in mg			Mean % recovery	% RSD	Mean % recovery	% RSD	Mean % recovery	% RSD
LOQ	20.3	0.0005	0.00049	101.5	1.49	103.8	1.21	98.5	1.08
50%	21.4	0.019	0.02	107	1.59	96.03	1.43	103.06	2.9
80%	20.9	0.03	0.03	104.6	0.38	96.16	0.73	98.9	1.96
100%	20.5	0.039	0.03	102.8	0.35	97.5	0.17	96.8	0.77
150%	20.3	0.05	0.05	101.7	0.26	98.1	0.26	96.2	1.26
200%	20.2	0.078	0.08	101.3	0.15	97.8	0.17	101.6	0.56

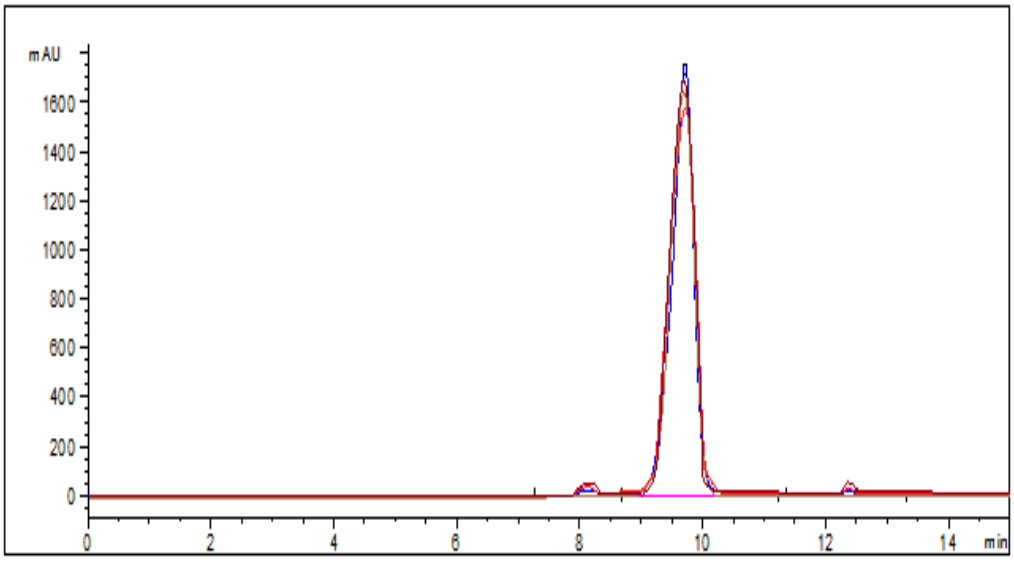

Figure 2:Overlap Chromatogram for accuracy and its impurities

Stability: Stability was observed at 25ºC and 8^0C. Similarity factor at 24 hrs were found to be 1.01, 1.00 and 1.00 for thalidomide, impurity A and impurity B under refrigerator temperature respectively.

. **Table 4:Stability of test preparation at ambient temperature about (25ºC)**

Time in hours	TM				Percentage of impurity A present in TM				Percentage of impurity B present in TM			
	% content		Difference from Initial		% Impurity		Difference from Initial		% Impurity		Difference from Initial	
	Sol-1	Sol-2			Sol-1	Sol-2			Sol-1	Sol-2		
Initial	97.56	102.23	NA		0.202	0.198	NA		0.17	0.161	NA	
24	98.22	102.506	0.66	0.203	0.201	0.201	0.001	0.003	0.18	0.172	0.01	0.011
Sol = solution; NA = not applicable												

Table 5:Stability of test preparation at refrigerator temperature about (8ºC)

Time in hours	TM				Percentage of impurity A present in TM				Percentage of impurity B present in TM			
	% content		Difference from Initial		% Impurity		Difference from Initial		% Impurity		Difference from Initial	
	Sol-1	Sol-2			Sol-1	Sol-2			Sol-1	Sol-2		
Initial	97.40	99.09	NA		0.21	0.19	NA		0.19	0.21	NA	
24	96.49	98.79	0.91	0.3	0.19	0.18	0.02	0.01	0.18	0.2	0.01	0.01
Sol = solution; NA = not applicable												

FORCE DEGRADATION STUDIES:

Forced degradation studies reports shown little deviation inThalidomide.. Percentage deviation of forced degradation studies was mentioned in table6.

Table 6: Forced degradation data

Stress conditions	Parameters	Thalidomide Area	% Degradation
Un stressed	Normal conditions	43410	0
Acid stressed	0.1N HCl/60°C/4hr	29952	29.9
Base stressed	0.1 N NaOH/60°C/4hr	35175	18.7
H2O2 stressed	3% H_2O_2/RT/4hr	34031	20.6
Thermal stressed	105°C/12hr	43026	1.2
Humidity	90%RH/ 7hr	43315	0.8

6. CONCLUSION:

A Simple and sensitive HPLC method for the determination of thalidomide and its impurities has been successfully developed and validated. The proposed method is simple, accurate, precise and highly sensitive .Hence this method can be used for routine analysis in pharmacy.

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