DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION AND QUANTIFICATON OF IMPURITIES IN IMIQUIMOD

ABSTRACT

New stability indicating reverse phase high performance liquid chromatography (HPLC) method is developed for the determination of Imiquimod and its impurities. Separation is achieved on a C18 column (Inertsil-ODS3 4.6 × 250 mm, 5 µm) using gradient elution mode with mobile phase-A having disodium hydrogen phosphate buffer (10mM) with 0.1 % v/v triethyl amine and pH adjusted to 6.0 with ortho-phosphoric acid while mobile phase-B consist of an equal mixture of methanol and acetonitrile. The flow rate was optimized to 1.2 mL/min and column oven temperature 30°C. Detection was carried out at wavelength 226 nm. This developed method is then validated as per International Council for Harmonisation (ICH) guideline and found out to be linear, accurate, specific, selective, precise, and robust. The drug is also subjected to forced degradation using stress conditions of acid-base hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found out only in harsh condition of oxidative degradation where degradation impurity is also predicted. All degradation products were well separated. Test solution was found to be stable for 24 hrs. The method can be successfully applied for the determination of Imiquimod and its impurities in routine and stability samples.

Keywords: Imiquimod, impurities, HPLC method, development, validation

1. INTRODUCTION

Imiquimod is described chemically as {1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine}. It is associated with imidazoquinolone family [1] and a nonnucleoside heterocyclic amine (Fig.1) which belongs to a class of products known for immune response modifiers use in the treatment for external genital warts [2]. The present work describes in this paper is related to the development and validation of a gradient reverse phase stability indicating HPLC method which is capable of determination of Imiquimod and its impurities in drug substances.

There are five major impurities reported for Imiquimod these are Impurity-A, Impurity-B, Impurity-C, Impurity-D and Impurity-E as given in Fig.1 with their chemical name given in Table 1. A thorough literature survey revealed that there is no any single method for determination of all these five impurities in Imiquimod [3-6]. Out of these five methods, three are used for quantification of Imiquimod [3-5] while another two methods[6-7] does not cover all the five impurities. Hence, the goal was set and achieved accordingly. The developed method provides better separation when compared to the previously existing methods [3-7] for determination of Imiquimod its impurities and degradation product.

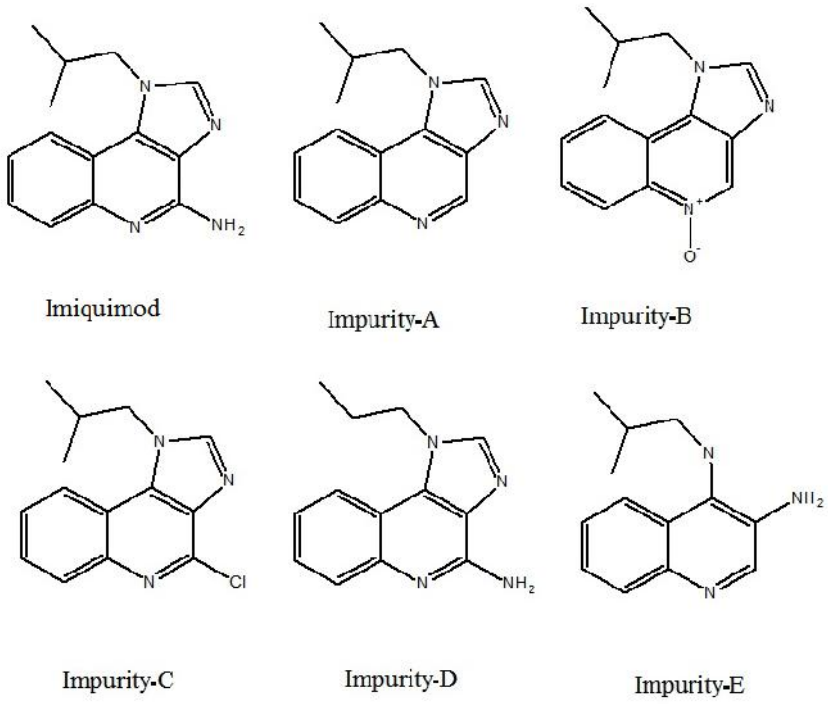


Fig. 1. Structure of Imiquimod and its impurities

Table 1. Chemical name of Imiquimod and its impurities

Component	Chemical name	Limit
Imiquimod	{1-(2-methyl-propyl)-1H-imidazo[4,5-c]quinolin-4-amine}	Not applicable
Impurity-A	1-Isobutyl-1*H*-imidazo[4,5-c]quinoline	0.15 %
Impurity-B	1-Isobutyl-1*H*-imidazo[4,5-c]quinoline 5-oxide	0.15 %

Impurity-C	4-Chloro-1-isobutyl-1*H*-imidazo[4,5-c]quinoline	0.15 %
Impurity-D	1-Propyl-1*H*-imidazo[4,5-c]quinolin-4-amine	0.15 %
Impurity-E	N-Isobutylquinoline-3,4-diamine	0.15 %

2. EXPERIMENTAL

2.1 Reagent, chemicals and standards

Imiquimod sample used for development and validation of analytical method (Purity 99.0%) was received from Indoco research centre, Navimumbai India, while Imiquimod, Impurity-A, Impurity-B, Impurity-C, Impurity-D and impurity-E were purchased from United States pharmacopeia (USP). Acetonitrile and methanol of HPLC grade were purchased from Merck (India). Disodium hydrogen phosphate, o-phosphoric acid (85%w/w) and Triethylamine were purchased from qualigens fine chemicals. Imiquimod and Impurities standard were of there greatest purity, given in Table 3.

2.2 Instrumentation

Waters, Alliance 2695 series HPLC system with photodiode array (PDA) 2998 and ultraviolet (UV) 2487 detectors was used for separation and detection. Data acquisition and calculations were carried out using Waters Empower software. Measurement of pH was done on Eutech pH meter (USA). For weighing of materials, Sartorius analytical balance was used. Water used for thought development and validation study was from the Milli-Q system (Millipore, Germany).

3. METHODOLOGY

3.1 Development and optimizing chromatographic condition

HPLC column such as Hypersil BDS C18, Inertsil C8 and Inertsil ODS-3 (Japan) of various dimensions were used with various buffers such as ammonium acetate, sodium octane sulfonate and disodium hydrogen phosphate in both isocratic and gradient elution mode for separation of Imiquimod and its impurities. The best separation was achieved on Inertsil ODS-3 (GL Science Part No. 5020-01732, Japan) column with a length of 250 mm and internal diameter 4.6 mm, packed with 5 μm particle size. The gradient elution mode as given in Table 2 was applied for the separation using mobile phase-A as 10 mM disodium hydrogen phosphate buffer with 0.1 % v/v triethylamine and pH of 6.0, while mobile phase-B consist of an equal mixture of acetonitrile and methanol. The mobile phase flow rate was optimized to 1.2 mL/min and injection volume to 20μL. The column temperature was kept at 30°C ± 2°C and the all peaks were monitored at wavelength 226 nm. Relative retention time (RRT) and relative response factor (RRF) with respect to Imiquimod for each impurity is calculated and reported in Table 3.

Table 2. Gradient elution program

Time (min)	Mobile phase-A (%)	Mobile phase-B (%)
0	65	35
5	65	35

20	45	55
30	45	55
35	65	35
40	65	35

Table 3. Potency of standard and there RRF & RRT

Standard	Relative Retention time (RRT)	Relative response factor (RRF)	Purity of standard
Imiquimod	1.00	1.00	99.8 %
Impurity-A	1.15	1.19	100.0 %
Impurity-B	0.54	0.65	99.0 %
Impurity-C	1.45	0.90	99.0 %
Impurity-D	0.71	1.12	97.0 %
Impurity-E	0.84	0.40	99.0 %

3.2 Preparation of solutions

Mobile Phase-A
Disodium hydrogen phosphate buffer solution of 10 mM was prepared in water with 0.1 % v/v triethylamine and adjusted the pH of this solution to 6.0 ± 0.05 with dilute phosphoric acid. Filtered and degassed by sonication.

Mobile Phase-B
Prepared by mixing equal volume of acetonitrile and methanol in a ratio of 50:50 (v/v)

Diluent
Prepared by mixing acetonitrile, water and phosphoric acid in a ratio of 500:500:0.5 (v/v/v) respectively.

Standard Stock solution (Imiquimod)
A standard stock solution of 0.25 mg/mL was prepared by weighing 25 mg of Imiquimod standard in 100 ml volumetric flask, dissolved in diluent and made upto volume.

Standard Stock solution (Impurities)
A standard stock solution of 0.25 mg/mL was prepared by weighing 25 mg of each impurity standard in 100 ml volumetric flask separately, dissolved in diluent and made upto volume.

Standard Solution

Prepared standard solution by transferring 0.15 mL standard stock solution (Imiquimod) to 100 mL volumetric flask and made upto volume with diluent.

Sample Solution
A sample solution of 0.25 mg/mL was prepared by weighing 25 mg of Imiquimod sample in 100 ml volumetric flask, dissolved in diluent and made upto volume.

4. RESULT AND DISCUSSION

The developed method is subjected to analytical method validation, which is conducted according to the International council for Harmonisation (ICH) guidelines [8-13]. First relative response factor was determined for each impurity with relative to Imiquimod. The parameter with which analytical method is validated is specificity, limit of detection, limit of quantitation, linearity, accuracy, precision, robustness and solution stability. Imiquimod sample was also subjected to stress studies in order to prove that the method is stability indicating and has the power to resolve all the degradant peaks from Imiquimod and its known impurities.

4.1 Specificity

Specificity is the capability of the method to measure the analyte response in the presence of its impurities. Each impurity was spiked in the test sample at their limit level and analysed. Imiquimod and its spiked impurity peaks were well separated from each other. There was no interference from peaks due to blank. Peak purity of Imiquimod and its impurities were passing for spiked test sample solution (Fig. 2 and Table 4).

Table 4. Peak purity of Imiquimod and its impurities

Peak name	Purity angle	Purity threshold	Peak purity
Imiquimod	0.121	0.266	Pass
Impurity-A	0.266	0.425	Pass
Impurity-B	0.434	0.551	Pass
Impurity-C	0.240	0.298	Pass
Impurity-D	0.320	0.385	Pass
Impurity-E	0.331	0.432	Pass

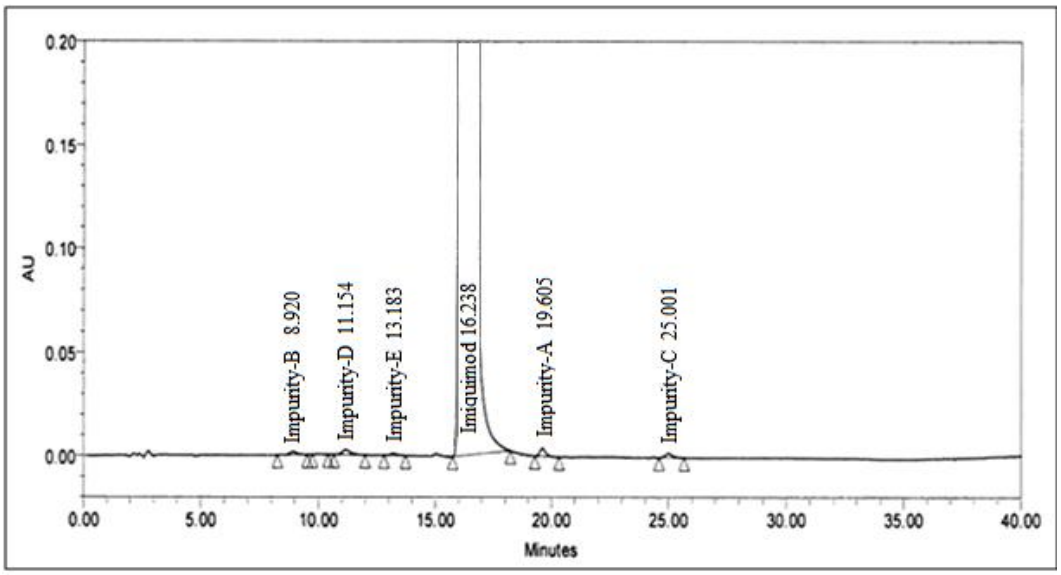


Fig. 2. Imiquimod sample spiked with impurities (specificity)

4.2 Limit of detection and quantitation

A series of standard solutions were prepared from standard stock solution in the range of 50% to 150% for Imiquimod and its impurities and injected. The residual standard deviation of the regression line and slope were calculated and limit of detection (LOD) and limit of quantitation (LOQ) was determined. LOD and LOQ calculated were well below 0.021% for all the impurities and Imiquimod (Table 5).

Table 5. Limit of detection and quantitation

Parameter	Imiquimod	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E
LOD (%)	0.007	0.002	0.002	0.001	0.003	0.007
LOQ (%)	0.021	0.007	0.008	0.003	0.009	0.021

4.3 Linearity

Series of linearity solution of Imiquimod and its impurities solution were prepared from the limit of quantification (LOQ) to 150%. Linearity curves were drawn by plotting the peak areas against the concentration of linearity solution for Imiquimod and its impurities. Regression coefficient, slope and % y intercept are reported. The observed regression coefficient was greater than 0.999 and % y intercept was less than 5.0% (Table 6 and Fig. 3).

Table 6. Linearity for Imiquimod and Impurities

Linearity	Imiquimod	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E
Regression	0.9999	0.9998	0.9995	0.9998	0.9998	0.9974
% y intercept	0.60	0.49	0.28	0.92	0.83	2.57
Slope	306825	374679	205885	277286	307827	120836
% Lower range	0.0245	0.0073	0.0080	0.0030	0.0090	0.0203
% Upper range	0.2306	0.2375	0.2248	0.2295	0.2250	0.2289

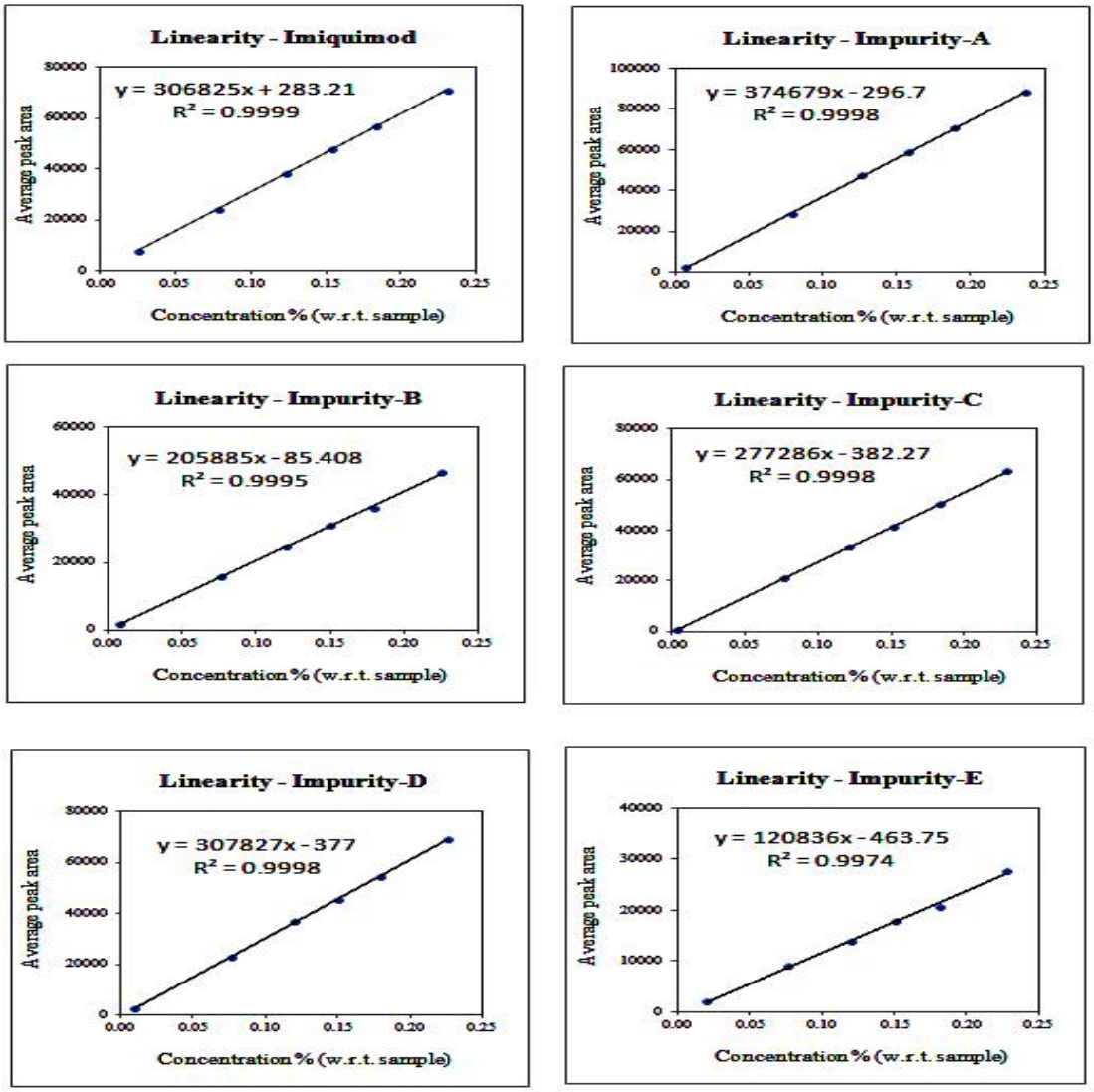


Fig. 3. Linearity curve for Imiquimod and its impurities

4.4 Precision

System precision was carried out by analysing six standard solutions of Imiquimod at a limit level concentration (0.15%). Relative standard deviation for the peak area of Imiquimod was calculated and found to be 2.39 %.

Precision at LOQ solution was prepared at the LOQ concentration level of Imiquimod and its impurities and injected six times. Relative standard deviation for the peak area of Imiquimod and its spiked impurities was less than 5.0 % (Table 7).

For repeatability and intermediate precision (different day different system), prepared six different test solutions with spiking the impurities at a limit level concentration (0.15%). Injected these solutions and calculated relative standard deviation (RSD) for impurity content. RSD observed for both the sets was less than 5.0 % (Table 7).

Table 7. Precision for Impurities

% RSD	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E
Precision at LOQ	0.62	4.17	2.61	1.81	1.10
Repeatability	3.60	0.00	0.00	0.00	0.00
Intermediate Precision	0.00	0.00	4.06	4.08	3.78

4.5 Accuracy

Accuracy of method was established by performing the recovery studies of each impurity. Impurities were spiked at LOQ, 80%, 100% and 120% in Imiquimod test sample in triplicate and analysed for its recovery. Recovery for each impurity obtained was between 80% and 120% (Table 8).

Table 8. Recovery of impurities

% Recovery	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E
LOQ	83.56	84.60	83.26	90.11	85.38
80%	90.78	95.65	93.30	82.73	92.20
100%	90.93	97.91	92.48	83.78	92.25
120%	81.55	97.62	94.31	84.62	89.15

4.6 Robustness

For robustness, four deliberate changes were done with respect to flow rate, column temperature, pH of buffer and concentration of buffer (Table 9). Each change consists of one upper set and one lower set. For each set, three preparations were done by spiking the impurities in the test sample at limit level and analysed. Relative standard deviation for spiked impurity content was observed, which was less than 5.0 % (Table 10).

Table 9. Robustness parameter changes

Sr.No.	Changes	Lower set	Upper set
Robustness-1	Mobile phase flow rate by 0.1 mL/min	1.1 mL/min	1.3 mL/min
Robustness-2	Column Oven Temperature by 5°C	25°C	35°C
Robustness-3	pH of buffer by 0.2 unit	5.8	6.2
Robustness-4	Buffer concentration by 10 %	9 mM	11 mM

Table 10. Robustness parameter changes

% RSD		Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E
Robustness-1	Upper set	4.03	0.00	0.00	0.00	3.77
	Lower set	0.00	3.69	0.00	0.00	0.00
Robustness-2	Upper set	0.00	0.00	0.00	0.00	4.03
	Lower set	4.22	0.00	0.00	0.00	3.94
Robustness-3	Upper set	0.00	3.77	0.00	4.03	0.00
	Lower set	3.69	3.77	7.14	4.68	0.00
Robustness-4	Upper set	0.00	3.46	3.77	0.00	0.00
	Lower set	0.00	0.00	3.94	0.00	0.00

4.7 Solution stability

Test solution stability was established by spiking the test sample with impurities at limit level and injected the same solution after every six hours time interval for 24 hours. Relative standard deviation for the content of spiked impurities, single impurity and total impurities was determined which was found out to be less than 5.0 %, thus solution stability was established up to 24 hours (Table 11).

Table 11. Solution stability of Imiquimod

Time	% Content (w.r.t. Sample)						
	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E	Single	Total
0 Hr	0.16	0.16	0.14	0.12	0.15	0.01	0.63
6 Hrs	0.16	0.16	0.15	0.12	0.15	0.01	0.64
12 Hrs	0.16	0.16	0.15	0.12	0.15	0.01	0.64
18 Hrs	0.16	0.16	0.15	0.13	0.15	0.01	0.64
24 Hrs	0.16	0.16	0.15	0.12	0.15	0.01	0.64
%RSD	**0.00**	**0.00**	**3.02**	**3.67**	**0.00**	**0.00**	**0.70**

4.8 Forced degradation studies

To determine whether the analytical method is stability-indicating, Imiquimod drug was subjected to stress degradation under various conditions. Intended degradation was attempted with stress conditions of photolytic degradation as per ICH, whereas acid hydrolysis was carried out by using 5N HCl and refluxed at 60-70°C for 3 hours, Alkaline degradation was carried out by hydrolysis using 5N NaOH solution and refluxed at 60-70°C for 3 hours, oxidative degradation was done by 30% H_2O_2 refluxed at 60-70°C for 3 hours and for the thermal degradation sample was heated at 105°C for 24 hours. There was no any significant degradation observed in all stress conditions except oxidative degradation (Table 12).

Table 12. Forced degradation of Imiquimod

Condition	% Impurity content							Observation
	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Impurity-E	Single	Total	
Test	0.02	BDL	BDL	BDL	BDL	BDL	0.02	No degradation
Thermal	0.03	BDL	BDL	BDL	BDL	BDL	0.03	No degradation
Acid	0.04	0.03	BDL	BDL	BDL	0.05	0.12	No degradation
Alkaline	BQL	BDL	BDL	BDL	BDL	0.01	0.01	No degradation
Hydrolysis	0.02	BDL	BDL	BDL	BDL	BDL	0.02	No degradation
Oxidative	0.16	BDL	BDL	BDL	BDL	13.24	15.71	Degradation
Photo	0.02	BDL	BDL	BDL	BDL	BDL	0.03	No degradation

BDL= Below detection limit, BQL = Below quantitation limit

Imiuqimod drug does not get degraded even with very harsh stress condition in acid, alkali, photolytic and thermal degradations. The only impurity formed is in the oxidative degradation (Fig.4.) is at retention time 10.889 min (RRT 0.62) which was subjected to the LCMS using acetic acid as mobile phase-A and keeping all chromatographic parameters and column same on Thermo Finnigan LCQ Advantage mass spectrometer using atmospheric pressure ionization (API) Source. Major, fragment obtained was m/z 541 which does not correspond to any probable structure. Further fragmentation on MS/MS gave m/z 257 fragment. The most probable structure predicted for this degradant is N-oxide impurity of Imiquimod (Fig.5).

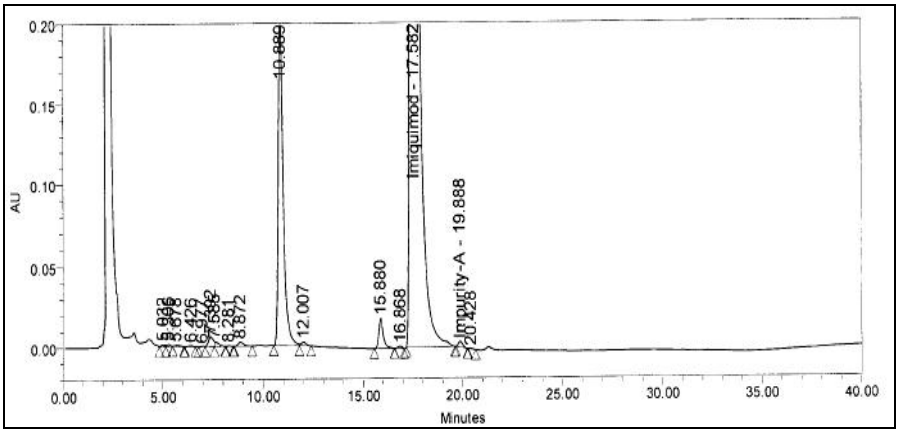
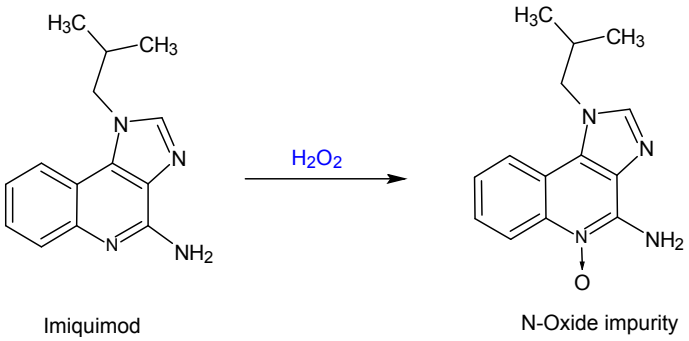


Fig. 4. Imiquimod oxidative degradation



Imiquimod N-Oxide impurity

Fig. 5. Imiquimod oxidative degradation impurity

5. CONCLUSIONS

The reverse phase HPLC method is developed for quantitative determination of impurities of Imiquimod. This method is validated and found out to be linear, accurate, precise, robust, specific and stability indicating. Acceptable data for all method validation parameters tested and found out to be satisfactory. Also the results of the stress testing of the drug, undertaken according to the ICH guidelines, revealed that the only in oxidative degradation impurity is formed which is confirmed by mass spectrometry as Imiquimod oxide impurity while with other degradation conditions, Imiquimod does not get degraded. The developed method can suitably use by quality control department to determine the impurities in commercial and stability test samples of Imiquimod.

6. ACKNOWLEDGMENT

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