Research Article

Development and Validation of UV Spectroscopy and RP-HPLC Methods for Estimation of Imiquimod

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ABSTRACT

The aim of present study is development and validation of two analytical methods for estimation of Imiquimod in API, Method A: UV Spectroscopy and Method B: RP-HPLC method. Imiquimod is a prescription medication that acts as an immune response modifier presently commercially available as Aldara® 5% imiquimod cream, approved by U.S. Food & Drug Administration ("FDA") in 1997. It is approved for external anogenital warts caused by human papillpma virus (HPV), actinic keratosis (AK) i.e. skin conditions of face and scalp, and superficial basal cell carcinoma (sBCC) a type of skin cancer. Drug showed linearity in the concentration range of 0.25-2.5µg/ml with correlation coefficient 0.996 and 0.05-0.55µg/ml with correlation coefficient 0.995 by UV Spectroscopy and RP-HPLC method respectively. Wavelengths, 245nm and 227 nm were chosen for analysis by Method A and Method B respectively. Different parameters such as linearity, range, precision, accuracy, ruggedness and robustness, limit of detection (LOD) and limit of quantification (LOQ) were used for validation in both the methods. The results were found to be acceptable as per the guidelines of International Conference on Harmonization (ICH). Both the methods are found to be novel, rapid, linear, precise, accurate, robust and rugged and can be successfully applied for the routine analysis of imiquimod. The methods are also found to be useful and economical.

Keywords: Imiquimod, UV Spectroscopy, RP-HPLC, Validation, ICH, Relative Standard Deviation (RSD).

INTRODUCTION

This research paper relates to development and validation of UV Spectroscopy and RP-HPLC methods for estimation of Imiquimod in API. The API or drug imiquimod is 1-isobutyl-1H-imidazo[4,5-c]-quinolin-4-amine, also known as 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine. It is a prescription medication that acts as an immune response modifier presently commercially available as Aldara® 5% imiquimod cream, approved by U.S. Food & Drug Administration ("FDA") in 1997. Imiquimod is generic and available globally under many brands as in 2015. Its molecular formula is $C_{14}H_{16}N_4$ with average molecular weight 240.3036. It is almost white crystalline powder, freely soluble in oleic acid and lactic acid with melting point 292-294°C and pKa value 2.7.[1-3] **Chemical structure** of Imiquimod is given below:
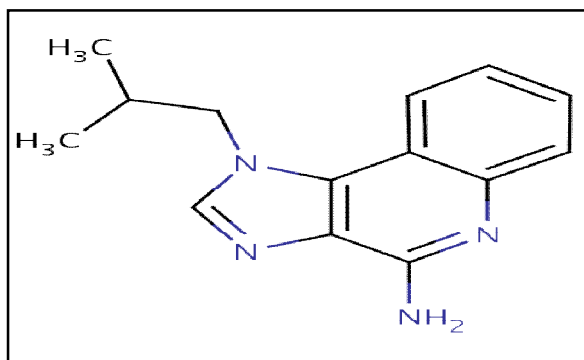
Imiquimod (trade name Aldara™) belongs to group of nucleoside analogs, a small molecule of imidazoquinoline family that was first synthesized as potential antiviral agent. It is used topically to treat warts on the skin of genital and anal areas universally.[4] Genital warts are the infections caused by one or more types of 100 recognized human papillomaviruses (HPVs).[5] Imiquimod helps to relieve and control wart production but does not cure warts, and new warts may appear during treatment. It does not fight the viruses that cause warts directly, however, also used to treat skin conditions of face and scalp called actinic keratoses and certain types of skin cancer called superficial basal cell carcinoma. At present, it is approved for external anogenital warts, actinic keratosis (AK), and superficial basal cell carcinoma (sBCC).[4]

Literature survey reveals (Ryan DF, 2005, Daniel DP, 2008, Madhududan Bachute T, 2013 and Ramineni SK 2013) determination of Imiquimod by HPLC and UV Spectroscopy methods.[6-9] However, as to our best knowledge, none of the methods UV Spectroscopy or HPLC has been validated in all the parameters for determination of this drug. Hence, the aim of present investigations is to develop and validate simple, rapid, accurate, economical and convenient analytical methods for determination of Imiquimod in bulk and topical dosage form suitable for regular quality control analysis.

Validation can be defined as (ICH) Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.[10] Data obtained

from method validation can be used to evaluate the quality, consistency and reliability of analytical method. It is an indispensable part of any good analytical practice.

The Necessity for Analytical Methods to be Validated or Revalidated:

- Before its introduction into routine use.

- Whenever the conditions change for which the method has been validated

- Whenever the method is changed outside the original scope of method.

- Furthermore revalidation may be necessary in the following **changing situations**:

- synthesis of drug molecule;

- constitution of finished product;

- analytical process.

The degree of revalidation required depends on type of changes. Validation may also be required due to convinced other changes.

Advantages of Analytical Method Validation:

- It results in level of confidence to the developer as well as to the user.

- The validation process might be a costly and time consuming exercise but results are found to be inexpensive and lead to better time management in the end.

- It also eliminates annoying repetitions

- It helps to absorb the shock of minor changes in analytical setup due to unavoidable reasons such as supplier or grade of reagent, and pays more than spent on the analytical method validation.[11]

The **objective** of analytical procedure proposed should be clearly understood since this will govern the validation parameters which need to be evaluated.

Typical validation parameters to be considered are Linearity, Concentration Range, Accuracy, Precision (Repeatability, Intermediate Precision), Ruggedness, Robustness, Limit of Detection and Limit of Quantification.[12,13]

MATERIALS AND METHODS

Method A: UV Spectroscopy

Materials: Imiquimod, the drug was supplied by Glenmark Pharmaceuticals Ltd, Mumbai. Methanol was obtained from Finar chemicals Ltd., Ahmadabad, India. The UV Spectrophotometer instrument used here was Shimadzu UV 1800, Japan.

Methods

Selection of solvent: Solubility of drug was checked in different solvents and UV spectra recorded. Absorbance

of drug exhibited distinct λmax in methanol and 0.1N HCl.[14]

Determination of absorption maxima: Standard solution 1mg/ml of Imiquimod was prepared in methanol and 0.1N HCl. From this solution, 10µg/ml concentration of drug was prepared. These solutions were scanned in UV region (200-400 nm) to determine λmax of drug.[15]

Preparation of stock and working standard solutions for pure drug: 10mg of imiquimod was weighed accurately and transferred into a 10 ml volumetric flask containing methanol. The solution was sonicated for 5 minutes and volume made up with further quantity of methanol to get 1mg/ml stock solution of Imiquimod.

This solution was further diluted with methanol to get working standard solution of 10µg/ml of drug.[16]

Preparation of sample solutions: Working standard solution of 10µg/ml of drug was further diluted with methanol to get 0.25-2.5µg/ml of drug by serial dilution method.

Method B: Reverse Phase High Performance Liquid Chromatography

Materials: Imiquimod was supplied by Glenmark Pharmaceuticals Ltd, Mumbai. Acetonitrile was obtained from Fischer Scientific, Mumbai. Ortho phosphoric acid and Triethyl amine were obtained from Thomas baker (Chemicals) Pvt. Ltd. Mumbai, India. All other reagents used were of analytical grade. The HPLC instrument used here was LC 2010 Shimadzu, Japan.

Methods

Chromatographic Conditions

Mobile phase: Consists of 10 Mm monobasic phosphate having 0.1% v/v triethylamine adjusted to pH 2.45 adjusted with O'phosphoric acid and acetonitrile (70:30v//v)

Absorption maxima: 227 nm

Injection Volume: 20µl

Column: C-18, dimension 250 × 4.6 mm, 5 µ from Phenomenex

Flow rate: 1.4ml/min.

Preparation of diluent: 0.1 N HCl and acetonitrile in the ratio7:3 (v/v) was mixed to prepare the diluent.

Preparation of standard stock solution: 10mg accurately weighed drug was put into 10ml volumetric flask containing 5 ml of diluent and sonicated for 10 mins. Then the volume was adjusted with more diluent up to the mark.

Preparation of sample solutions: Sample solutions of different conc. from 0.05, 0.1., 0.2, 0.3, 0.4, 0.5 µg/ml were prepared from above stock solution and diluted with mobile phase.

Parameters of Validation

Linearity and Range: The linearity of an analytical method is its ability to elicit test results which are directly proportional to analyte concentration in samples within a given range. The range of an analytical method is the interval between upper and lower analyte concentration in sample including these concentrations for which it has been established that the analytical method has a suitable level of linearity, accuracy and precision. To establish the linearity and range of proposed methods, various aliquots of standard solution of drug were prepared from stock solution and analyzed. Sample solutions of drug with different conc. from 0.25-2.5µg/ml and 0.05-0.5 µg/ml were analyzed by UV spectrophotometer using methanol as blank at 245 nm and HPLC at 227 nm respectively. Their absorbance/area measured.

Precision: Precision studies were carried out to establish the repeatability and reproducibility of proposed methods by using six replicates of same concentration of sample solution.

Repeatability: It was determined by preparing six replicates of same concentration (1 µg/ml and 0.5µg/ml in UV Spectroscopy and HPLC method respectively) of sample and their absorbance/area measured.

Reproducibility: Intraday precision study was carried out by preparing drug solution of same concentration (1 µg/ml and 0.5µg/ml in UV Spectroscopy and HPLC method respectively) and analyzing it at three different times in a day. The same procedure was followed for three different days to determine interday precision. The results were indicated as %RSD.

Accuracy: Accuracy of a method is the degree to which observed results correspond to true value of analyte in the sample. The determination was done at three different levels (80%, 100%, and 120% of sample concentration) .Three samples of each level were prepared and total 9 determinations done as per ICH conditions. The samples were analyzed and their absorbance/area measured and results indicated as % RSD.

Robustness/ Ruggedness: Robustness/ Ruggedness of an analytical procedure are measure of its ability to remain unaffected by small changes in method parameters and provide an indication of its reliability.

Ruggedness: Ruggedness was determined by carrying out analysis by two different analysts at the same operational and environmental conditions. The respective absorbance/ area noted and results indicated as % RSD.

Robustness: To check the robustness of proposed methods: analysis was carried out at two different temperatures, room temperature and at 18°C and respective absorbance measured (UV Spectroscopy method). The results were indicated as % RSD.

Similarly analysis was carried out at three different wavelengths and flow rates and respective area measured (HPLC method). The results were indicated as % RSD.

LOQ AND LOD: Limit of detection (LOD) is the minimum quantity of analyte in sample that can be detected. Limit of quantification (LOQ) is the minimum quantity of analyte in sample that can be quantitatively determined by suitable precision and accuracy. LOQ and LOD were determined using equations: LOQ-10σ/S and LOD-3.3σ/S where σ is the standard deviation of response and S slope of related calibration curve.

RESULTS AND DISCUSSION

Determination of absorption maxima and Selection of wavelength: The UV spectra obtained by scanning solution of Imiquimod in methanol and 0.1N hydrochloric acid are shown in Figure 1 and 2 respectively. The wavelength at which maximum absorbance occurred was selected as absorption maxima (λmax). The drug showed λmax at 245 nm in methanol. Therefore, λmax 245 nm was used for further studies by UV spectrophotometer as good linearity was observed at this wavelength. But, the drug showed λmax at 227 nm and 245 nm in 0.1N hydrochloric acid. The shift of λmax to 227 nm represents protonation of Imiquimod in acidic media.

Absorption maxima 227nm was used for HPLC analysis as it gave sharp peak as compared to 245nm. It was not possible to get separate peak at 245 nm. Some of the chromatograms obtained are shown in Figure 3. The average retention time is observed to be 3.05 mints.

Linearity and Range: Drug shows linearity in the concentration range of 0.25-2.5µg/ml and 0.05-0.5µg/ml by UV Spectroscopy and HPLC methods respectively. Linearity of data given in Table 1 was found by the plot of concentration verses absorbance (UV Spectroscopy method) and concentration verses area measured (HPLC method). The standard calibration curves obtained for both methods are given in Figure 4 and 5. Correlation coefficient obtained from standard curve is 0.996 and 0.995 by UV Spectroscopy and HPLC method respectively.
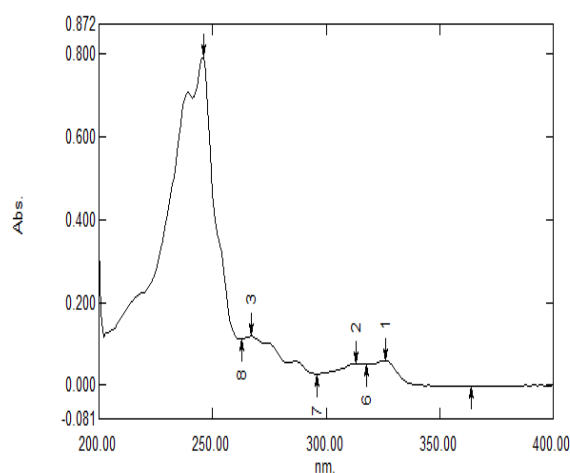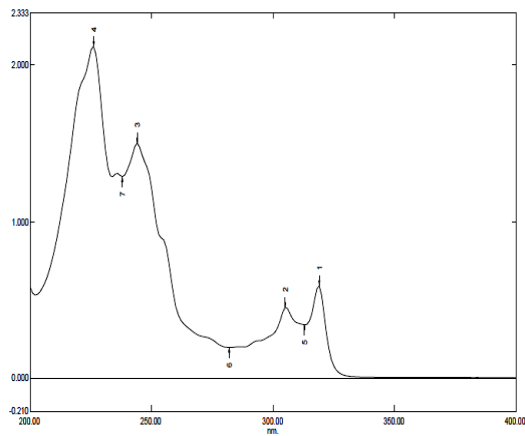

Figure 1: Scanned UV Spectra of Imiquimod in Methanol
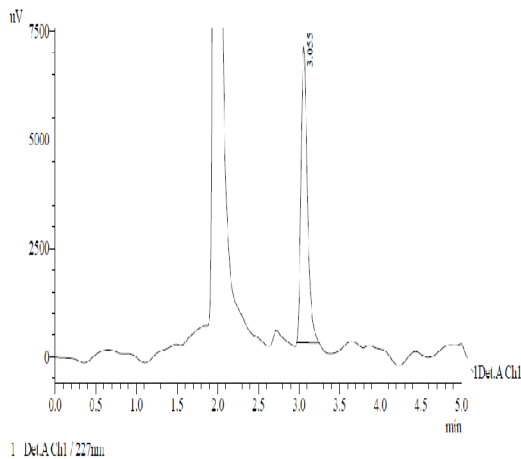
Figure.2: Scanned UV Spectra of Imiquimod in HCl

Figure 3: Chromatogram of Imiquimod (IMQ)

Table 1: Linearity data of Imiquimod

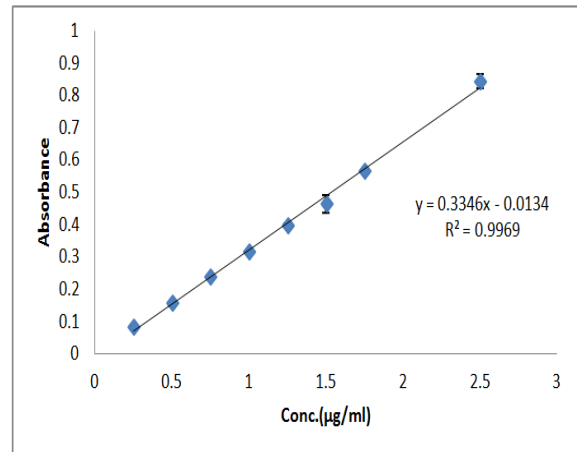| Method	Concentration (µg/ml)	Absorbance/Area	Statistical Analysis
UV Spectroscopy in methanol	0.25	0.0853±0.003	**Intercept** = 0.013
	0.50	0.157±0.0052	**Slope** = 0.334
	0.75	0.238±0.0062	**Straight line equation**
	1.00	0.3173±0.0066	y = 0.334x - 0.013
	1.25	0.399±0.0043	
	1.50	0.4653±0.027	
	1.75	0.5656±0.0061	
	2.50	0.8436±0.022	
HPLC	0.05	33697.9 ± 55.08	**Intercept** = 24502
	0.1	37247.16 ± 24.58	**Slope** = 14686
	0.2	55267.56 ± 51.20	**Straight line equation**
	0.3	66490 ±228.14	y = 14586x + 24502
	0.4	82654.06 ± 119.79	**Regression coefficient**
	0.5	99303.56 ± 75.16	

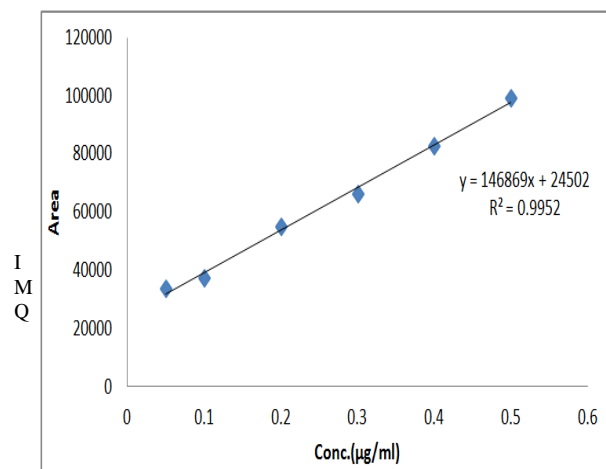**Figure 4:** Standard plot of Imiquimod by UV Spectroscopy in methanol at 245nm

Figure 5: Standard curve of Imiquimod by RP-HPLC

Precision: The precision results show good repeatability (Table 2) with percent relative standard deviation less than 2. Results calculated as %RSD values for intraday and interday precision studies are also shown in the same table and found to be satisfactory.

Table 2: Results of Precision

Method Precision	UV Spectroscopy (%RSD)	RP-HPLC (%RSD)
Repeatability	1.05	0.326
Intraday precision		
1	1.9	0.326
2	1.6	0.341
3	1.2	0.964
Interday precision		
Day 1	1.12	0.341
Day 2	1.05	0.584
Day 3	1.53	0.633

Accuracy: The results of accuracy are shown in Table 3. Total 9 determinations were done at three different levels of sample concentration (80%, 100%, and 120%). The results calculated as % RSD are found to be satisfactory.

Ruggedness: The results of ruggedness determined by carrying out analysis by two different analysts are indicated as % RSD shown in Table 3. The values are found to be less than 2.0.

Robustness: Robustness of both methods was determined by performing same analysis at slightly different parameters from the optimized conditions of selected method. The results indicated as %RSD are shown in Table 3 and are found to be below 2.0%.

LOQ AND LOD: The values of LOQ and LOD are found to be 0.28 and 0.094µg/ml, and 0.00104 and 0.000343 by UV Spectroscopy and HPLC method respectively indicating sensitivity of methods.

The results of various validation parameters of developed methods are shown in Table 3.

Table 3: Summary of results for methods developed and validated for determination of Imiquimod

Method Parameter	UV Spectroscopy	HPLC
Absorption maxima	245 nm	227nm
Linearity range	0.25-2.5 µg/ml	0.05-0.5 µg/ml
Regression equation	Y=0.334x+0.013	Y=14686x+24502
Slope (b)	0.334	14686
Intercept (a)	0.013	24502
Correlation coefficient	0.996	0.995
Precision (%RSD) Repeatability Intraday Interday	1.05 1.62 1.23	0.326 0.543 0.513
Accuracy (%RSD) 80% 100% 120%	1.19 1.38 1.46	0.113 0.280 0.156
Ruggedness (%RSD) Analyst 1 Analyst II	1.59 1.33	0.053 0.64
Robustness (%RSD) Room temperature Temperature 18 °C Wave length 224 nm Wave length 227 nm Wave length 230 nm Flow rate 1.2ml/min Flow rate 1.4ml/min Flow rate 1.6ml/min	0.734 1.56	1.97 0.334 0.466 0.686 0.334 0.910
LOQ (µg/ml)	0.28	0.00104
LOD (µg/ml)	0.094	0.000343

CONCLUSION

The proposed methods UV Spectroscopy and RP-HPLC are found to be simple, accurate, economical and convenient for the analysis of Imiquimod in API. Linearity is obeyed in the concentration range of 0.25-2.5µg/ml with correlation coefficient 0.996 and 0.05-0.5µg/ml with correlation coefficient 0.995 by UV Spectroscopy and HPLC method respectively. The methods are precise as % RSD values for repeatability; interday and intraday are found to be less than 2. Accuracy of proposed methods determined by recovery studies and good % recovery (80-120%) of drug obtained indicates that the methods are accurate. The methods are also found to be rugged and robust as % RSD values are less than 2. The limit of detection and limit of quantification of proposed methods are found to be 0.094 and 0.28µg/ml and 0.000343 and 0.00104µg/ml by UV Spectroscopy and HPLC method respectively indicating that the methods developed are sensitive. Hence, concluded that both methods can be used for routine analysis of Imiquimod.

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