ORIGINAL ARTICLE

Determination and quantification of imiquimod and its related impurities from bulk drug production by high-performance liquid chromatography

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Abstract

Aim: A simple, selective, precise, and inexpensive reversed-phase high-performance liquid chromatography method has been developed and validated for the determination of imiquimod and related impurities such as 4-Chloro-1-isobutyl-1H-imidazo[4,5-c] quinoline, 1-isobutyl-1,5-dihydro-imidazo[4,5-c] quinolin-4-one, 1-isobutyl-4-methoxy-1H-imidazo[4,5-c] quinoline, and N-(1-isobutyl-1H-imidazo[4,5-c]quinolin-4-yl)-hydroxylamine-O-sulfonic acid. **Materials and Methods:** The method was followed using spectrophotometric detection at 260 nm with a Phenomenex Luna-RP-C18 column (250 × 4.6 mm, 5 µm) at a flow rate of 1.0 mL/min. The mobile phase consisted of deionized water spiked with 1% H_3PO_4 (Solvent A) and acetonitrile (Solvent B). **Results and Discussion:** Imiquimod and the common four impurities were eluted at different time intervals from 10 to 20 min. The developed method was validated in terms of system suitability, specificity, precision, linearity, accuracy, limit of detection, and limit of quantification. **Conclusion:** The mobile phase composition of 1% v/v H_3PO_4 in water: acetonitrile (90:10 v/v), showed good separation and resolution. Therefore, the proposed analytical procedure could be useful for regular monitoring, pharma manufacturing labs, and research scholars.

Key words: High-performance liquid chromatography, imiquimod, impurity analysis, limit of quantification, method validation

INTRODUCTION

The drug imiquimod – chemically1-isobutyl-1H-imidazo [4,5-c]- quinolin-4-amine,alsoknownas1-(2- methylpropyl)-1Himidazo[4,5-c]quinolin-4-amine is widely used as a topical cream to treat warts on the skin of genital and anal areas.[1,2] Imiquimod belongs to group of nucleoside analogs, as a small molecule of imidazoquinoline family that was first synthesized as potential antiviral agent.[3] This drug is a strong stimulator of the immune cells, interferon-α (IFN-α), interleukin-6, and tumor necrosis factor-α being the major cytokines elicited in response to the drug.[4,5] Topical application of imiquimod elevates the constriction of cytokines, including the essential cytokine for antiviral response, IFN-α; this being a preliminary event of an immunological cascade resulting in the stimulation of innate immune as well as cell-mediated pathway of adaptive immunity.[6] This immune change mediates an indirect antiviral, antiproliferative, and antitumor pastime of imiquimod *in vivo* (ref). The imiquimod is an amazing medication for genital warts.[7-9] There has been a suggestive evidence that imiquimod, when applied to skin, leads to the activation of cells of Langerhans, which then migrate to nearby lymph nodes to set off an adaptive immune response.[10] Natural killer cells, macrophages, and B-lymphocytes are the types of cells naturally triggered by imiquimod recent studies suggest that imiquimod exerts its effect through growing levels of the opioid growth factor receptor none.[11] The study revealed that blocking function

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of OGFr with siRNA technology resulted in lack of any antiproliferative outcome of imiquimod.[12-15] Imiquimod drug is hence a drug of great value in nosocomial setting and treatment regimen.

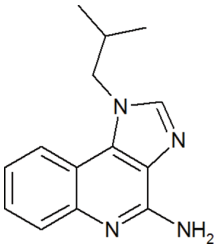
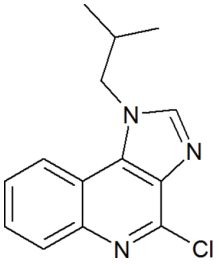
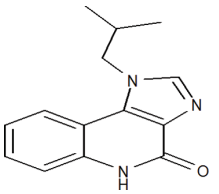
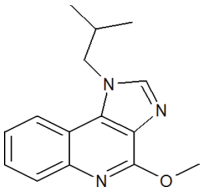
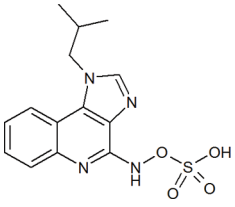
So far, various methods including thin-layer chromatography (TLC) and ultra-performance liquid chromatography (UPLC) have been described for the determination of imiquimod and its impurities. However, using TLC and UPLC, there is no literature available in the simultaneous analysis of imiquimod and its four impurities in bulk drug. Therefore, in the present study, we have developed a simple, accurate, sensitive, and validated reversed-phase high-performance liquid chromatography (RP-HPLC) method for separation and determination of imiquimod and related impurities. Furthermore, the method has been optimized and validated as per the International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

Materials

Imiquimod and impurities were procured from Sigma-Aldrich, India. HPLC grade solvents (water and acetonitrile) were obtained from Merck India, pvt., Ltd. All other chemicals and reagents used were of analytical grade. The molecular structure of imiquimod is listed in Table 1.

Table 1: Molecular information of imiquimod and four impurities

Molecule/ compound	IUPAC name	Molecular structure	Molecular formula	Molecular weight
Imiquimod	1-Isobutyl-1H-imidazo[4,5-c]quinoline-4-ylamine		$C_{14}H_{16}N_4$	240.31
Impurity 1	4-Chloro-1-isobutyl-1*H*-imidazo[4,5-c]quinoline		$C_{14}H_{14}ClN_3$	259.74
Impurity 2	1-Isobutyl-1,5-dihydro-imidazo[4,5-c]quinolin-4-one		$C_{14}H_{15}N_3O$	241.30
Impurity 3	1-isobutyl-4-methoxy-1H-imidazo[4,5-c]quinoline		$C_{15}H_{17}N_3O$	255.32
Impurity 4	N-(1-isobutyl-1H-imidazo[4,5-c]quinolin-4-yl)-hydroxylamine-O-sulfonic acid		$C_{14}H_{16}N_4O_4S$	336.37

Chromatographic Conditions

The analysis was performed with Shimadzu HPLC with LC-20AT pump SPD-20A interfaced with LC solution software. The system equipped with a binary solvent manager, autosampler, and column heating compartment, and UV detector. This system was controlled by the LC solution software. The analytes were separated on 250 × 4.6 mm i.d., 5 µm particle size Luna-RP-C18 (Phenomenex) column.

The mobile phase consisted of 1% H_3PO_4 in water (Solvent A) and acetonitrile (Solvent B). Separation was carried out using gradient elution method. Before analyses, the mobile phase was filtered through syringe filter and then degassed ultrasonically for 10 min. For chromatographic separation gradient was set up with initial concentration of 10% Solvent B, it was then linearly programmed to 70% Solvent B in 25 min and held for 5 min. The mobile phase condition was returned to the starting solvent mixture in 1 min. The system was allowed to equilibrate for 10 min before the next injection. The analyses were conducted at a flow rate of 1.0 ml/min. Column temperature was maintained at 30°C with injection volume of s 20 µL. The compounds were scanned in the range of 200–400 nm using photo diode array (PDA) detector and the absorption max was found at 260 nm.

Validation of the Method Parameters

The method performance was evaluated for different parameters, namely, system stability, system suitability, specificity and selectivity, linearity, precision, intermediate precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).[16-20]

System Suitability

The test solution was prepared by adding 50.07 mg of test substance in 10 ml of Solvent A in a 50 ml volumetric flask, the volume was further made up with Solvent B. The reference solution (a) was made by accurately weighed 10.02 mg of reference standard in a 100 ml volumetric flask and dissolved in 10 ml of diluent A and make up with the same solvent up to the mark. 1 ml of above reference solution (a) was taken into a 100 ml volumetric flask and dilute to volume with diluent B (0.1% with respect to test solution).

Preparation of Reference Solution (b) for System Suitability

Accurately weighed 10.09 mg of impurity 2 standard in a 100 ml volumetric flask and dissolved in 10 ml of diluent A and make up with the same solvent up to the mark. 1 ml of above reference solution (b) was taken into a 100 ml volumetric flask and diluted to volume with diluent B.

System Suitability Test

The resolution between the peaks of imiquimod and impurity 2 obtained more than 1.5 using reference Solution (b). The reference Solution-b was injected onto an HPLC column and calculated the resolution between the peak of imiquimod and impurity 2 and injected 6 times of reference Solution-a and recorded the chromatograms. Six replicates of the readings were taken into account to calculate average and standard deviation. The RSD of reference standard areas is <3%.

Specificity and Selectivity

The specificity of the method was determined by injecting the individual solutions of A.I (1 µg/mL) and impurities (1 µg/mL) and peak purity was checked. The specificity was determined by comparing the chromatogram of the blank run to the chromatogram of the single impurity run. Thereafter, a solution containing a mixture of impurities and imiquimod were injected.

Preparation of Solutions for the Analysis

In 10 mg of the imiquimod reference standard, impurity-1, impurity-2, impurity-3, and impurity-4 were taken into a different 100 mL volumetric flasks and dissolved in diluents-A and made up to the mark with the same solvent. Further 1.0 mL of above each solution was transferred into different diluted to 100 ml with Solvent B and the resulting solutions were injected into HPLC system. Peak purity was determined for imiquimod and related impurities, results were plotted as a mean of six independent readings for determining the selectivity.

Linearity

Different known concentrations of imiquimod and their impurities (0.2–2.0 µg/mL) were prepared in diluent-A by diluting the stock solution. Standard solutions were injected and peak area was measured. A calibration curve has been plotted for concentration of the standards injected versus area observed and the linearity of the method was determined from the correlation coefficients.

Preparation of Linearity Solutions

The imiquimod and its impurities standard stock solutions were individually prepared in diluent-A at a concentration level 100 µg/mL and stored in a freezer at -18°C until further use. The stock standard solutions were used for up to 3 months. Concentrations (0.2, 0.5, 1.0, 1.5, and 2.0 µg/mL) of working standards were prepared from the stock solutions by dilution using Solvent A. The serial dilution details are presented in Table 2.

Precision

Solution preparation

10 mg of imiquimod, impurity-1, impurity-2, impurity-3, and impurity-4 weighed into six different 100 mL volumetric flasks and 20 mL of diluent-A was added to dissolve the content and made up to the mark with the same solvent. The precision of method was calculated by injecting above six sample solutions of imiquimod, impurity-1, impurity-2, impurity-3, and impurity-4 into HPLC and the relative standard deviation (RSD) was calculated.

Intermediate precision was assessed by analyzing six replicates of the HPLC runs for each sample prepared on different days.

Accuracy

The accuracy was determined as prescribed by ICH guidelines.[12] Known quantities of impurities spiked into imiquimod at 50–100–150% of the nominal limit of 0.10% for each impurity. The analysis was performed in triplicate at each level.

Preparation of test solutions

100 µg/ml standard linearity solution was used as standard stock solution for accuracy 0.5 ml, 1 ml, and 1.5 ml of above standard stock solution were taken into a three different 100 ml volumetric flasks and make up with dissolution diluent-B up to the mark. These solutions were used for checking accuracy.

LOD and LOQ

LOD and LOQ were assessed in accordance with ICH guidelines.[12] The method chosen was based on signal-to-noise ratio. Following formulae were taken into consideration for obtaining LOD and LOQ values using the following formulas:

$$LOD = \frac{3 \times s}{S} \qquad LOQ = \frac{10 \times s}{S}$$

Table 2: Serial dilution of linear standard solutions

Stock solution concentration (µg/mL)	Volume taken from the stock solution (mL)	Final make-up volume (mL)	Final concentration (µg/mL)
100	0.2	10	2.0
100	0.15	10	1.5
100	0.1	10	1.0
2	2.5	10	0.5
2	1.0	10	0.2

Calculations

The imiquimod impurities were determined by comparison of peaks areas with the following formula:

$$\text{Percentage Imiquimod impurity} = \frac{At \times C \times D \times PS}{Ar \times W\ \text{sample} \times Fc} \times 100$$

Where:
At: Peak area of impurity obtained by test solution
Ar: Peak area of imiquimod obtained by reference solution (a)
C: Imiquimod concentration in reference solution (a) (mg/ml)
D: Sample dilution (ml)
W sample: Sample weight in test solution (mg)
PS: Purity of reference standard
Fc: Response factor of impurity.

$$\%\ \text{Recovery} = \frac{\text{Recovered concentration}}{\text{Fortified concentration}} \times 100$$

RESULTS

System Suitability

The resolution between the peaks of imiquimod and impurity 2 is more than 1.5 and % RSD (full form of RSD) of reference standard areas was found to be <3% on each day of analysis. Hence, the system suitability passes the acceptance criteria. The suitability of method was confirmed by verifying the USP parameters such as retention times (RT), theoretical plates (N), tailing factors (T), RSD, and resolution (R). The results are presented in Table 3.

Specificity and Selectivity

Aliquots of standard solution, impurity spiking sample solution, diluents, and mobile phase solvents were assayed to check the specificity. There was no to interfere of main peaks in the chromatogram, as shown in Figure 1. The peak purity was more than 99% for imiquimod and impurities for verified the specificity of the method.

Table 3: System suitability parameter results

Product	Retention time in min	R	N	T
Impurity 1	20.5	9.1	189154	1.26
Impurity 2	13.5	32.1	132541	1.41
Impurity 3	18.7	11.5	165878	1.32
Impurity 4	16.1	12.8	89574	1.08
Imiquimod	9.2	-	95687	1.13

Linearity

The correlation coefficients were above >0.99 at wavelength of 260 nm for imiquimod and their impurities. The results are mentioned in Table 4. Calibration curves are depicted in Figure 2 while representative chromatogram is presented in Figure 3.

Intermediate Precision

The precision (%RSD) and exact weighing details of imiquimod, impurity-1, impurity-2, impurity-3, and impurity-4 are presented in Tables 5-9.

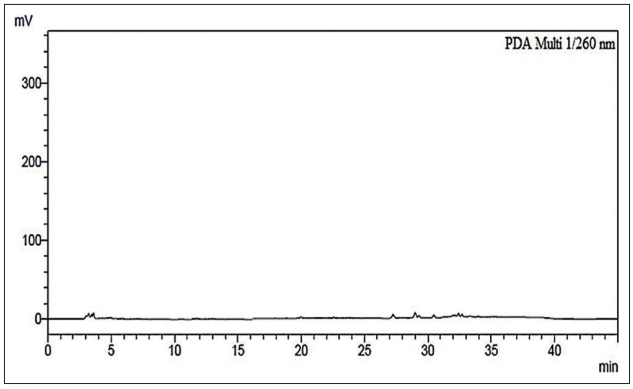


Figure 1: Representative chromatogram of diluent as mobile phase (blank)

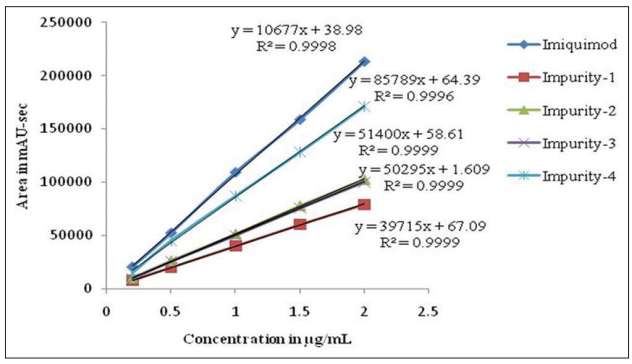


Figure 2: Linear regression curve of imiquimod and its four impurities

LOD and LOQ

Intermediate precision was assessed by analyzing six replicates of test solutions prepared on different days, using fresh mobile phase, and a different operator as in precision. The results are given in Table 3-7.

Accuracy

The results were obtained within acceptable limits. The representative chromatogram is shown in Figure 4 and results are presented in Table 10.

LOD and LOQ

The LOD and LOQ are established successfully for each impurity in Imiquimod based on signal-to-noise ratio method (10, 11). The results are presented in Table 11.

DISCUSSION

A simple, inexpensive, and precise HPLC method was successfully developed for identification and quantification of impurities from imiquimod bulk drug API. In this method, it was carried out by using 250 × 4.6 mm i.d., 5 µm particle size Luna-RP-C18 (Phenomenex) column. Injection volume of 20µl is injected and eluted with the mobile phase consists of 1% H_3PO_4 in water (Solvent A) and acetonitrile (Solvent B), which is pumped at a flow rate of 1.0 ml/min with column and sampler temperatures at 30°C and ambient respectively and runtime was optimized to 40 min. Detection was carried out at 260 nm. The peaks obtained were sharp with RT of 20.5 min for impurity-1, 13.5 min for impurity-2, 18.7 min for impurity-3, 16.1 min for impurity-4, and 9.2 min for imiquimod. The results obtained were accurate and reproducible. The method estimation was statistically validated in terms of selectivity, accuracy, linearity, precision, and robustness. For selectivity, the chromatograms were recorded for standard and sample solutions of imiquimod

Table 4: Linearity data of imiquimod and its four impurities

Range (%)	Concentration in µg/mL	Area mAU-sec imiquimod	Area in mAU-sec impurity-			
			1	2	3	4
20	0.2	20,874	7712	9954	9521	14,919
50	0.5	52,651	19,985	26,124	25,741	45,074
100	1	109,521	39,954	51,254	50,178	87,121
150	1.5	158,965	60,185	77,854	75,846	128,158
200	2	213,395	79,015	102,385	100,254	171,152
Slope		106,771.37	39,714.52	51,399.61	50,294.61	85,788.86
Intercept		38.98	67.10	58.61	1.61	64.39
Correl		0.9998	0.9999	0.9999	0.9999	0.9996

Table 5: Intermediate precision of impurity-1

Injection	Weight in mg	Area in mAU.Sec	Day	Results	
1	10.11	40,745	Day 1	Average	41108.67
2	10.23	41,611			
3	10.24	41,314		STDV	555.84
4	10.15	40,778		RSD	1.35
5	10.27	41,087			
6	10.17	40,331			
7	10.12	42,521	Day 2		
8	10.03	41,088			
9	10.01	41,001			
10	10.16	40,859			
11	10.19	41,265			
12	10.11	40,704			

Table 6: Intermediate precision of impurity-2

Injection	Weight in mg	Area in mAU.Sec	Day	Results	
1	10.17	50,172	Day 1	Average	50539.42
2	10.15	51,112			
3	10.09	50,002		STDV	533.22
4	10.19	51,014		RSD	1.06
5	10.03	49,965			
6	10.14	50,147			
7	10.13	51,211	Day 2		
8	10.11	50,087			
9	10.07	50,004			
10	10.12	50,621			
11	10.16	50,789			
12	10.21	51,349			

Table 7: Intermediate precision of impurity-3

Injection	Weight in mg	Area in mAU.Sec	Day	Results	
1	10.27	51,874	Day 1	Average	50701.75
2	10.24	51,789			
3	10.16	50,147		STDV	832.35
4	10.13	50,075		RSD	1.64
5	10.26	50,901			
6	1017	50,457			
7	10.31	52,344	Day 2		
8	10.14	50,455			
9	10.18	50,214			
10	10.13	49,963			
11	10.19	50,078			
12	10.15	50,124			

and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore, the method is selective for the determination of related substances in imiquimod. Current method can be used for the estimation

Table 8: Intermediate precision of impurity 4

Injection	Weight in mg	Area in mAU.Sec	Day	Results	
1	10.11	86,741	Day 1	Average	86978.33
2	10.17	86,985			
3	10.19	87,254		STDV	518.36
4	10.21	87,305		RSD	0.60
5	10.23	87,405			
6	10.15	86,298			
7	10.16	86,889	Day 2		
8	10.19	86,954			
9	10.25	87,369			
10	10.31	87,954			
11	10.17	86,391			
12	10.12	86,195			

Table 9: Intermediate precision of imiquimod

Injection	Weight in mg	Area in mAU.Sec	Day	Results	
1	10.11	107,602	Day 1	Average	107667.92
2	10.18	108,121			
3	10.16	108,024		STDV	287.84
4	10.12	107,652		RSD	0.27
5	10.14	107,712			
6	10.17	107,789			
7	10.16	107,712	Day 2		
8	10.18	107,815			
9	10.10	107,311			
10	10.12	107,356			
11	10.18	107,795			
12	10.08	107,126			

Table 10: Recovery results of imiquimod impurity

Limit %	Impurity-1 in %	Impurity-2 in %	Impurity-3 in %	Impurity-4 in %
50	99.2	94.2	92.3	95.2
50	98.5	95.1	91.7	95.7.
50	99.7	94.8	92.7	94.9
100	99.2	93.9	93.2	95.1
100	96.6	94.7	92.7	96.2
100	96.7	95.4	93.2	97.4
150	100.1	99.2	96.7	98.2
150	99.9	98.9	96.7	99.2
150	99.8	99.1	98.2	99.5

Table 11: Limit of quantification and limit of detection results of imiquimod impurities

Impurity	Average S/N	Concentration in µg/mL
LOD		
1	3	0.003
2	4	0.01
3	3	0.003
4	3	0.06
LOQ		
1	10	0.01
2	11	0.05
3	11	0.01
4	10	0.02

of imiquimod in biological fluids, accelerated stability experiments, and ability to separate the drug from their degradation products. The chromatogram developed has well resolved peaks of imiquimod and its common impurities without any interference. The separation method provides

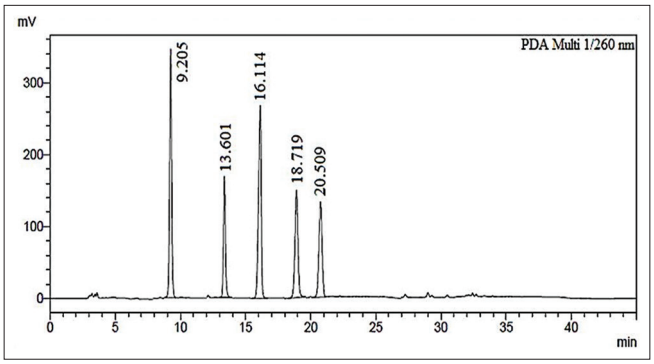


Figure 3: Representative chromatogram of linearity standard – 4.5 µg/mL

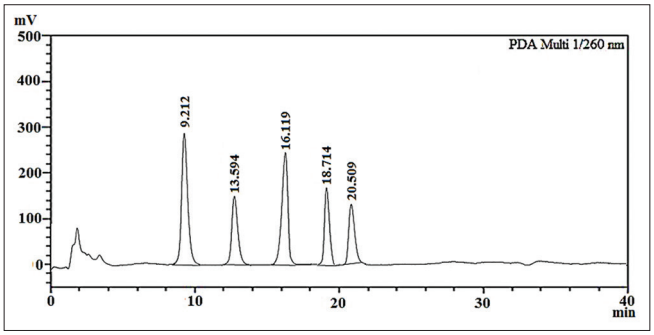


Figure 4: Representative chromatogram of 100% potrification level of impurities

acceptable recovery values for drug and impurities. From the results, we conclude the developed simple method can be used for the identification and quantification of imiquimod and its impurities in bulk drug industry with desired precision and accuracy along with high throughput.

CONCLUSIONS

The method developed in the present study for quantitative determination of imiquimod and its impurities is rapid, precise, accurate, and selective. The method was completely validated showing satisfactory data for all method – validated parameters tested. The mobile phase composition of 1% v/v H_3PO_4 in water: acetonitrile (90:10 v/v) showed good separation and resolution. Satisfactory validation parameters such as linearity, recovery, precision LOD, and LOQ were established by following ICH guidelines. Therefore, the proposed analytical procedure could be useful for regular monitoring, pharma manufacturing labs, and research scholars.

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