DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR THE SIMULTANEOUS ESTIMATION OF IMIQUIMOD AND THYMOQUINONE

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ABSTRACT:
A novel and sensitive high-pressure liquid chromatography (HPLC) method was developed for the simultaneous estimation of Imiquimod (IMI) and Thymoquinone (THY). Chromatographic elution was accomplished using Hypersil™ C-18 reverse phase column measuring 250 mm × 4.6 mm (5 µm) and mobile phase as Acetonitrile: Water (0.1% formic acid) in 65:35 ratio. The flow rate used in the method is 1.0 mL/minute and was observed at 249 nm employing PDA Detector. The run time of the analytical procedure was 16 minutes. The retention time was obtained at 4.1 min (IMI), and 14.3 min (THY). International Conference on Harmonization (ICH) guidelines were followed to validate the method and the parameter included a limit of detection (LOD), robustness, limit of quantification (LOQ), and system suitability. The regression graph plotted showed linearity from 10 to 100 µg/ml concentration.
All results are under acceptable limits and the method could be suitably employed for the simultaneous estimation of both drugs in quality control and assay.

Keywords: Imiquimod, Thymoquinone, HPLC, validation, simultaneous estimation

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1. INTRODUCTION

An Immunomodulator which is an imidazoquinolone synthetic derivative, Imiquimod is chemically known as 1-(2-methyl propyl)-1H-imidazo [4, 5-c] quinoline-4-amine (Figure 1). Imiquimod has the molecular formula $C_{14}H_{16}N_4$, is a crystalline white powder, and has a molecular weight of 240.30g/mol with a melting point of 292-294 °C. Imiquimod is readily miscible in oleic acid and lactic acid. It is well known for its antiviral and antitumor activity in animal models but not able to exhibit its antiproliferative action in cells. It acts by inducing the cytokines by binding to the toll-like receptor 7. In 1977, Food and Drug Administration (FDA) approved Aldara® 5% imiquimod ointment for the prophylaxis and treatment of genital warts, superficial basal cell carcinomas, and scratchy and scaly patches on the skin due to prolonged sun exposure known as actinic keratosis. (Imiquimod, 2022), (Sharma, Kumar, & Rana, 2020), (Gupta, Browne, & Blu, 2002).Indications of its potential therapeutic effect in the therapies of human papillomavirus warts and infection (HPV), squamous cell carcinoma, and vulvar intraepithelial neoplasia. (Gupta, Browne, & Blu, 2002), Bowen's disease (Stanley, 2002), cervical cancer (Frank, et al., 2020), as an age-defying agent (Metcalf, Crowson, Naylor, Haque, & Cornelison, 2006) is entrenched.

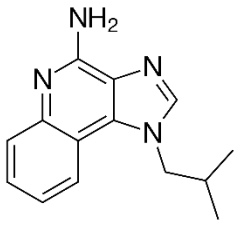


Figure 1: Chemical Structure of Imiquimod

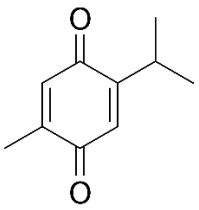


Figure 2: Chemical Structure of Thymoquinone

A monoterpene molecule Thymoquinone (THY) chemically named 2-Methyl-5-(propan-2-yl) cyclohexane-2,5-diene-1,4-dione (Figure 2) was extracted from plant Black cumin i.e., *Nigella sativa* (Ranunculaceae) by El-Dakhakhany in 1963 (Gali-Muhtasib, Roessner, & Schneider-Stock, 2006). Thymoquinone's molecular formula $C_{10}H_{12}O_2$, is a crystalline off-white coloured powder, with a molecular weight of 164.204 g/mol and a melting point of 45-47 °C. It is readily miscible in alcohol and ether. It induces apoptosis and regulates the signalling of both pro-apoptotic and anti-apoptotic genes, acting as a scavenger for free radicals. It has gained fame in the scientific associations due to its pharmacological activities ranging from anti-oxidant, anti-inflammatory, anti-histaminic, anti-microbial, anti-psoriasis, anti-neoplastic and immuno-modulatory. It has remarkable anticancer and cytoprotective properties on different cell lines such as the breast, Cervical, Pancreatic, Colon, Leukaemia, Buccal, Cranial & neck, Lungs, Skin, Ovarian, and Bladder (Muhammad , et al., 2018), (Gupta, Ghosh , & Gupta, 2016), (Gali-Muhtasib, Roessner, & Schneider-Stock, 2006), (Almajali, et al., 2021). It has also been indicated to have gastro-protective, hepatoprotective, nephroprotective, and neuroprotective attributes. It is also believed to have beneficial effects in the therapeutics of epilepsy, diabetes, cardiovascular disease, reproductive complications, and lung diseases, as well as bone complications, arthritis and fibrosis (Goyal, et al., 2017). Furthermore, a significant amount of research implies that Thymoquinone has very few side effects and no significant toxic effects (Darakhshana, Pour, Colagar, & Sisakhtnezhad, 2015).

Although both drug moieties have well-known effects in the area of cancer, thus, the author recommends that this combination to be studied further in depth. As combining naturally derived moiety with the synthetic one is taking us one step closer to the plant-based approach of medicine making it acceptable to accommodate alternative therapy, either showing synergism or antagonism with the conventional medicine because of the drug resistance and adverse effects. (Woo , Kumar , Sethi, & Tan, 2012). Conventional medicine is yet inadequate to offer complete eradication of cancer.

Many High-Pressure Liquid Chromatography (HPLC) Analytical methods are in existence for the estimation of Imiquimod (IMI) individually (Sharma, Sharma, Singh, & Katare, 2019) and in combination with other drug moieties (Tomar, Sharma, Kumar, Jain, & Ahirrao, 2021) has been reported in the literature. The analytical method was also reported in Biological matrices (Mu, et al., 2016), in nanoformulation (Frank, et al., 2020), (Remiro, et al., 2022), in bulk drug (Rao, Patrudu, Rao, & Ganesh, 2020) and in marketed preparation (Bachute & Turwale, 2013).

Additionally, several HPLC techniques have been developed for Thymoquinone (THY) individually

(Aboul-Enein & Abou-Basha, 1995), (Iqbal, Ahmad, & Pandey, 2018), in combination with other drug moieties (Jagtap, Mahajan, Parte, Pananchery, & Jain, 2021), (Soliman, et al., 2020), in biological matrices (Ahmed, Khan, & Alkharfy, Many analytical methods are published in the literature for IMI and THY independently, but no simultaneous estimation analytical method of IMI and THY together has been stated. As a result, the authors were inspired to create a trailblazing simultaneous estimation method for IMI and THY, which is intended to be used in assay studies and routine analysis due to its cost-effectiveness.

The current study aimed to create and validate a novel, smooth, quick, and parsimonious liquid chromatography method for the synchronal evaluation of Imiquimod and Thymoquinone in accordance with guidelines laid by ICH (ICH, 2005).

According to the literature, IMI has been quantified by HPLC at various wavelengths, such as 226 nm (Sharma, Kumar, & Rana, 2020), 242 nm (Frank, et al., 2020), 244 nm (Sharma, Sharma, Singh, & Katare, 2019), 245 nm (Bachute & Turwale, 2013) and 260nm (Rao, Patrudu, Rao, & Ganesh, 2020). Similarly, THY has also been quantified using an HPLC method at 249 nm (Alam, et al., 2022), 254 nm (Ahmed, Khan, & Alkharfy, 2015) (Gilani, et al., 2019), (Soliman, et al., 2020), 255 nm (Ansar, et al., 2020) and 295 nm (Aboul-Enein & Abou-Basha, 1995).

IMI and THY were detected at 244 nm and 255 nm, respectively, while developing the approach for simultaneous estimation. However, the isobestic wavelength was discovered to be 249 nm, allowing for efficient data analyzation in the combined quantification of two distinct drug moieties. Thus, using the previously mentioned optimised wavelength, a simplified precise method for simultaneous estimation of IMI and THY was developed, which was able to overcome the shortcomings of the previous individual methods. The method developed has also been substantiated in congruence with ICH guidelines.

2. MATERIAL AND METHODS

2.1. Materials

Imiquimod (IMI) was gifted sample from Glenmark Pharmaceuticals Ltd, Mumbai. Thymoquinone (THY) was procured from Sigma Aldrich, Mumbai. The supply of Water (HPLC-grade), Methanol and Acetonitrile (ACN) was by Merck Specialties Pvt. Ltd., India. The column

2015), along with Box-Behnken Design (BBD) (Alam, et al., 2022), and in nano formulation (Gilani, et al., 2019), (Ansar, et al., 2020), (Jain, et al., 2017).

used was HPLC Hypersil C-18 Column and was procured by Thermo Fisher, India; and the syringe filters were procured from Axiva Sichem Biotech, New Delhi, India. All other chemicals employed in the method development were of pharmaceutical or analytical grade.

2.2 Identification of Standard Drug:

Identification of the bulk drugs (IMI & THY) was executed by measuring the melting point, infrared spectroscopy, and solubility.

2.3 HPLC Instrument and Chromatographic Conditions

Analytical equipment used for this method development was Waters- e2695 Separation Module, along with Waters-e2998 PDA detector equipped with Software EMPOWER 3 for data acquisition and analysis. The stationary phase used was Hypersil C-18 Reverse Phase column measuring 250mm * 4.6 mm, 5 µm. ACN: Water (0.1% formic Acid) in a 65:35 ratio was used as mobile phase using isocratic elution mode at a temperature of 25ºC. Detection was done at 249nm using a PDA detector. The flow rate was maintained at 1ml/min with ambient column temperature. The volume of injection was 10 µl with a run time of 16 minutes.

2.3. Preparation of Standard and Working Solutions

IMI and THY standard solutions were formulated separately at 1000µg/ml concentration and labelled S1 and S2, respectively. Stock solutions of concentration 100µg/ml were made from the standard solutions (S1& S2) and labelled as A1 and A2. Using the mobile phase as a diluent, a working stock sample of varied concentrations (10, 20, 40, 60, and 100 µg/ml) was made from the stock solution (A1& A2). Furthermore, IMI-THY combination samples were prepared in the mobile phase at concentrations 10, 20, 40, 80, and 100 µg/ml for both drugs. All samples were kept in amber-coloured bottles in a freezer at 4ºC prior to HPLC analysis. Filtration of all the samples was done through a syringe filter (pore size: 0.22 µm). (Wrightson, Myers, & Galandiuk, 1995)

3. VALIDATION

Soon after the creation of an optimization technique, the method of simultaneous estimation

of IMI and THY was validated in accordance with ICH guidelines Q2 (R1) (ICH, 2005). HPLC methods should be validated using various parameters to ensure that the overall performance attributes of the strategy correspond to the demands of its desired uses only (Zothanpuii & Selvakumar, 2020). The parameters considered are system suitability testing, linearity, ruggedness, specificity, detection limit, and quantification limit (Tiwary et al. 2021).

3.1. System Suitability Test

This Suitability test is pivotal in liquid chromatography because it helps validate the testing method and ensures reproducibility. Suitability testing was performed in six replicates with a sample size of 20g/ml for both IMI and THY, with the RT and Peak area examined at a UV detection value of 249nm. As per US-FDA guidelines, the acceptable limit of relative standard deviation, %RSD is not more than 2%.

3.2. Specificity

While developing an analytical process, Specificity is the capacity to isolate standard drug from its combination such as other drugs, excipients or impurities. To assess specificity, individual solution chromatograms were compared to a blank solution and their combination. The blank solution lacked IMI and THY, but the remaining constituents and preparation method were exactly equivalent to the sample drug solution.

3.3. Linearity

Solutions of 10, 20, 40, 80, and 100 µg/ml concentration were prepared from standard solution for linearity determination. Standard curve was prepared from the values of peak area (Y-axis) and corresponding concentration (X-axis).
The linear equation was established from IMI, THY, and IMI-THY calibration curves that were plotted individually by arranging concentration and peak area.

3.4. Ruggedness

Ruggedness refers to the capability of producing an outcome under varying conditions, like by using variant investigator or same instrument of unsimilar make. We have used three distinct samples with concentration levels of 10, 20, and 30 µ g/ml which were analysed by two varied investigator using the same HPLC analytical equipment and on different HPLC by the same researcher in separate laboratory. Percentage amount concentration recovery for both analytes was calculated, and ruggedness findings from two separate investigators were compared.

3.5. Sensitivity

The sensitivity of the LC analytical method is determined by its ability to detect and quantify the minimal amount of drug sample i.e. LOD (limit of detection) and LOQ (limit of quantification).

	LOD	**LOQ**
Signal to noise (s/n) Ratio	3:1	10:1
Calculation Formula **S= S.D.** **s=Slope**	3.3 S/s	10S/s

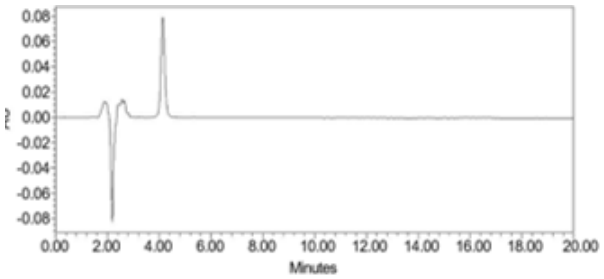
4. RESULT AND DISCUSSION

4.1. Development and Optimisation of the Method

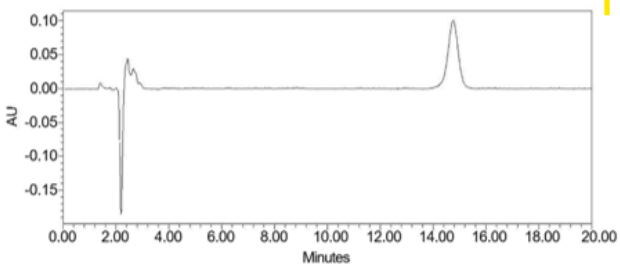
To make the prepared methodology more economically sustainable and reliable, an isosbestic wavelength was chosen for an analytical study that is predicted by observing the absorption maxima of IMI and THY at a particular wavelength. IMI and THY solutions having 10 µg/ml concentration each were analysed by UV Spectroscopy in the range of 400-200 nm.

IMI has the highest absorbance at 244 nm, followed by THY at 255 nm. A single wavelength of 249 nm was chosen based on spectral data and it was validated by a PDA detector. Mobile phase selection for method development was done among ACN/Methanol and Water in varied concentrations in isocratic and gradient flow.
The best isolation of IMI and THY was observed in an ACN: Water (0.1% Formic Acid) in a 65:35 ratio as mobile phase with a flow rate of the mobile phase in stationary column is 1ml/minute and the total run time of the analytical method was 16 minutes. IMI had a retention time of 4.1 minutes and THY had a retention time of 14.3 minutes. IMI and THY both were tested at their respective maxima, 244 and 255 nm, individually (Figs. 1a and b). The combination of IMI and THY was also analysed, and the peaks were separated at 4.1 and 14.3 min for IMI and THY, respectively, implying no change in the retention time of the individual and that combination (Fig. 1c).

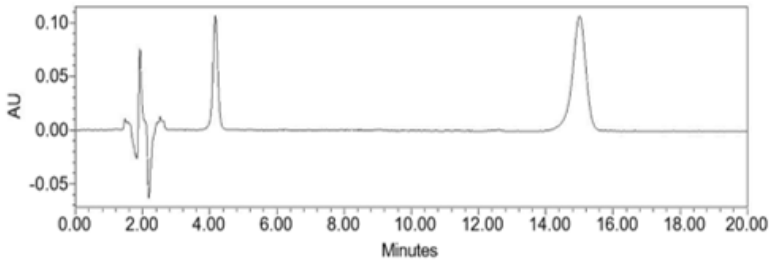
The developed method for evaluating IMI, THY, and their combinations suggests it to be economical, efficient, and can detect both analytes in only 16 minutes of run time. Thus, it proves its efficiency over other stated methods of estimation.



a) Chromatogram of Imiquimod at λmax 244nm



b) Chromatogram of Thymoquinone at λmax 255 nm

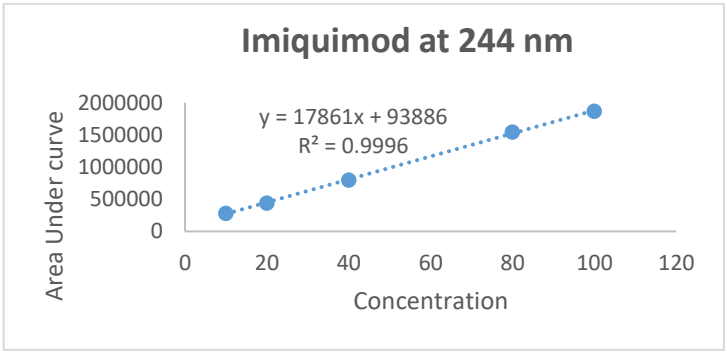


c) Chromatogram of Simultaneous determination of Thymoquinone and Imiquimod at isosbestic wavelength 249 nm.

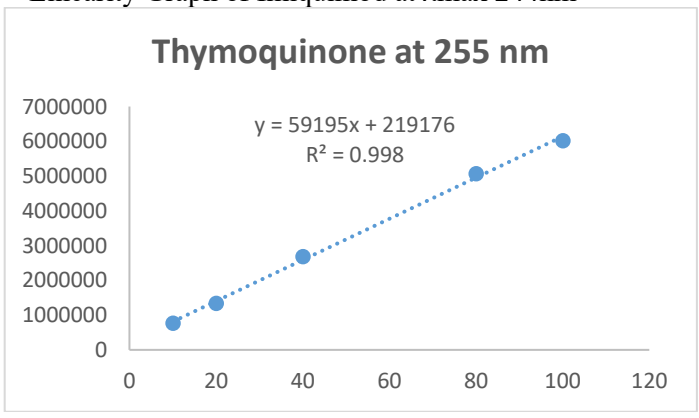
Figure 3: Chromatogram of Imiquimod, Thymoquinone and its combination at their respective wavelength.

4.2. Method Validation

4.2.1. Linearity

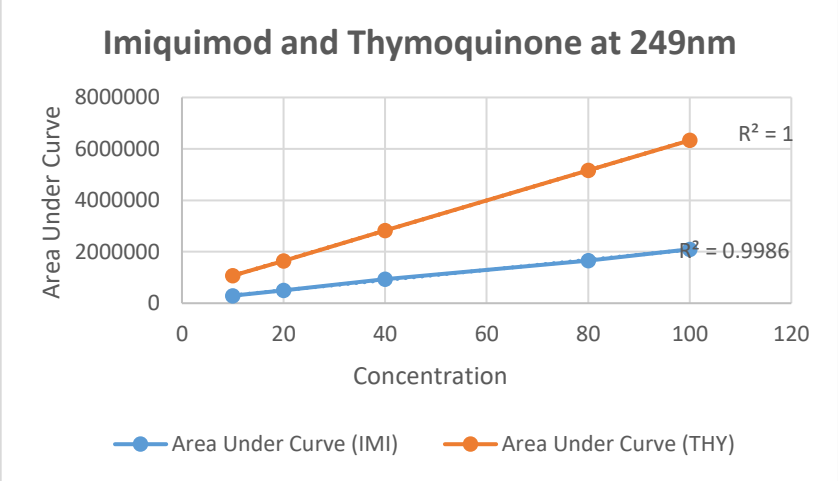


a) Linearity Graph of Imiquimod at λmax 244nm



b) Linearity Graph of Thymoquinone at λmax 255nm

c)



Linearity Graph of Simultaneous estimation of Thymoquinone and Imiquimod at isosbestic wavelength 249 nm

Figure 4: Linearity Graph of Imiquimod, Thymoquinone and its Combination at respective wavelengths**.**

Five distinct concentration levels of 10, 20, 40, 80, and 100 µg/ml were prepared as well as analysed for IMI, THY, and IMI -THY independently. IMI, THY, and IMI-THY were analysed at 244nm, 255 nm, and 249 nm, respectively, to determine the linearity of the Analytical method. A graph was created by plotting the concentration of the drug moiety on the X-axis with its comparative AUC on the Y-axis. The R_2 value for IMI at λmax 244 nm was 0.9996, for THY at λmax 255 nm was 0.998, and for IMI-THY at max 249 nm was 0.9986 for IMI and 1 for THY. (Fig 4). The above values demonstrated that the current analytical Methodology is linear in the defined concentration range.

4.2.2. System Suitability Test

a) System Suitability of Imiquimod at 249 nm		
System suitability criteria	**Retention Time**	**Peak Area Count**
1	4.12	413209
2	4.18	421378
3	4.06	408971
4	4.15	418107
5	4.11	407816
6	4.08	414271
Average	4.116666667	413958.6667
Standard deviation	0.044121046	5206.225146
Relative standard deviation (RSD)	1.07176629	1.25766787
b) System Suitability of Thymoquinone at 249nm		
System suitability criteria	**Retention Time**	**Peak Area Count**
1	14.34	1335501
2	14.26	1312634
3	14.38	1298632
4	14.36	1341783
5	14.39	1351352
6	14.42	1347929
Average	14.35833333	1331305.167
Standard deviation	0.055287129	21737.89004
Relative standard deviation (RSD)	0.385052555	1.632825485

Table 1: a) System suitability (R_t & Peak area Count) for Imiquimod at 249 nm;
b) System suitability (R_t & Peak area Count) for Thymoquinone at 249 nm.

Six IMI and THY sample runs were performed to determine the RSD of peak area count and retention time. The mean retention time for IMI was 4.11± 0.044 min with an RSD of 1.07% and 14.35 ± 0.055 min with an RSD of 0.38%. The average peak area of IMI was 413958.67± 5206.2 with an RSD of 1.25%, while the average peak area of THY was 1331305.167± 21737.89 with an RSD of 1.64%. (Table 1). All of the obtained RSDs for Peak Area count and retention time for both IMI and THY are less than 2 %, indicating that the current Analytical technique meets the criteria for suitability and reproducibility.

4.2.3. Specificity

The specificity of the current analytical method was evaluated by correlating chromatograms of an IMI, THY, and IMI -THY standard solution with a blank solution.

Each standard sample was injected in a volume of 10 µl and was analysed separately. IMI and THY retention times were observed to be 4.1 and 14.3 minutes, respectively, when examined separately. In the IMI-THY mixture, IMI and THY had retention times of 4.1 and 14.3 minutes, respectively (Fig 1). Thus, indicating the specific nature of the analytical method as the retention time remains constant whether the samples are evaluated individually or simultaneously.

4.2.4. Sensitivity

Sample	Slope of linear curve	Standard deviation (SD)	LOD	LOQ
Imiquimod at λmax 244 and Thymoquinone at λmax 255				
Imiquimod	17861	39961.14	7.383223896	22.37341
Thymoquinone	59195	126538.2	7.05424546	21.3765
Imiquimod & Thymoquinone at Isobestic λmax 249 nm				
Imiquimod	19748	59556.3	9.952187057	30.15814
Thymoquinone	58458	291580.7834	16.45996417	49.87868

Table 2: Sensitivity Table of Imiquimod and Thymoquinone individually and in combination at their respective wavelength

The sensitivity of the HPLC analytical method is assessed by examining the standard curve of the samples and combinations to ascertain the LOD i.e. lowest detection amount of the drug and LOQ i.e. lowest quantified drug value. Thus, the LOD for IMI at λmax 244 nm was calculated to be 7.38 ng/ml, and the LOD for THY at λmax 255 nm was 7.05 ng/ml. LOD at the isobestic wavelength of 249 nm was 9.95 ng/ml for IMI and 16.45 ng/ml for THY (Table 2).

LOQ is the minimum iota of drug sample that is assessed by the proposed method. It was estimated to be 22.37 ng/ml for IMI at λmax 244nm and 21.37 ng/ml for THY at λmax 255nm. The LOQ for said current anticipated analytical method at λmax 249 nm was 30.15 ng/ml for IMI and 49.87 ng/ml for THY (Table 2).

As a result, the LOD and LOQ statistics demonstrate an intriguing evaluation possibility for the current analytical method in assay procedures and formulation development.

4.2.5. Ruggedness

(A) Ruggedness Score of Imiquimod and Thymoquinone on different HPLC unit by same investigator					
Drug Sample	**Sample Concentration (µg/mL)**	**Amount Recovered**		**Recovery (%)**	
		HPLC-I	**HPLC-II**	**HPLC-I**	**HPLC-II**
IMI	**10**	9.61 ±0.68	9.89 ± 0.76	96.10	98.929
	20	19.55 ± 0.35	19.75 ± 0.37	97.77	98.7725
	30	29.68 ± 0.69	28.91 ± 0.87	98.95	96.37
THY	**10**	9.68 ± 0.42	9.64 ± 0.35	96.80	96.49
	20	19.41 ± 0.18	19.44 ± 0.08	97.06	97.24
	30	29.76 ± 0.65	29.33 ± 0.26	99.23	97.79
(B) Ruggedness Score of Imiquimod and Thymoquinone by the different investigator on same HPLC system.					
Drug Sample	**Sample Concentration (µg/mL)**	**Amount Recovered**	**Recovery (%)**		
		Investigator-I	**Investigator-II**	**Investigator-I**	**Investigator-II**
IMI	**10**	9.74 ± 1.55	9.85 ± 1.77	97.42	98.57
	20	19.43 ± 1.21	19.89 ± 0.77	97.19	99.48
	30	29.73 ± 1.46	29.41 ± 0.94	99.12	98.03
THY	**10**	9.85 ± 1.24	9.65 ± 0.41	98.51	96.53
	20	19.77 ± 0.25	19.55 ± 0.09	98.87	97.79

Table 3: Ruggedness Score of Imiquimod and Thymoquinone at λmax 249 nm

The robustness of the test was used to evaluate the reproducibility of the outcomes obtained on different HPLCs by the same investigator and on the same HPLC by different investigators. The test was then repeated on different HPLCs by the same analyst. The recovered concentration of IMI for sample concentration 10, 20 and 30 µg/ml at 249 nm came out to be 9.61 ± 0.68, 19.55 ± 0.35 and 29.68 ± 0.69 µg/ml along with percentage recovery of 96.10, 97.77 and 98.95 % respectively on HPLC-1 and the recovered concentration was 9.89 ± 0.76, 19.75 ± 0.37 and 28.91 ± 0.87 µg/ml with percentage recovery 98.92, 98.77 and 96.37 % respectively on HPLC -2. Similarly, the recovered concentration of THY by the same analyst for sample concentration 10, 20 and 30 µg/ml at 249 nm came out to be 9.68 ± 0.42, 19.41 ± 0.18 and 29.76 ± 0.65 µg/ml along with percentage recovery of 96.80, 97.07 and 99.23 % respectively on HPLC-1 and the recovered concentration was 9.64 ± 0.35, 19.44 ± 0.08 and 29.33 ± 0.26 µg/ml with percentage recovery 96.49, 97.24 and 97.79% respectively on HPLC -2. (Table 3(A)).

The ruggedness data was then collected by different analysts using the same HPLC (HPLC-1). The recovered concentration of IMI for sample concentration 10, 20 and 30 µg/ml at 249 nm came out to be 9.74 ± 1.55, 19.43 ± 1.21 and 29.73 ± 1.46 µg /ml along with percentage recovery of 97.42, 97.19 and 99.12 % respectively by analyst-1 and the recovered concentration was 9.85 ± 1.77, 19.89 ± 0.77 and 29.41 ± 0.94 µg/ml with percentage recovery 98.57, 99.48 and 98.03 % respectively by analyst-2. Similarly, the recovered concentration of THY for-sample concentration 10, 20 and 30 µg/ml at 249 nm came out to be 9.85 ± 1.24, 19.77 ± 0.25 and 29.62 ± 1.05 µg/ml along with percentage recovery of 98.51, 98.87 and 98.76 % respectively by analyst-1 and the recovered concentration was 9.65 ± 0.41, 19.55 ± 0.09 and 9.71 ± 0.70 µg/ml with percentage recovery 96.53, 97.79 and 99.05 % respectively by analyst-2.

The study results show a significant level of ruggedness, implying that the HPLC analytical method is experimentally verified on separate systems and by varied researchers. The results obtained from validation are congenial with ICH Guidelines. (Sharma, Goyal, & Chauhan, 2018), (Swartz & Krull, 1997)

CONCLUSION

The current research presents an innovative method for estimating IMI and THY simultaneously in assay procedures and process monitoring. The methodology is simple, rapid, highly selective, sensitive and precise, and it has been validated per ICH guidelines.

The method takes only around 16 minutes to complete. Thus, faster elution of the combination with high resolution saves researcher time and solvents, making the proposed method economical and quick for routine analysis. This method could be used to simultaneously estimate both analytes (IMI and THY) in formulation development, assay procedures and quality control checks.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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