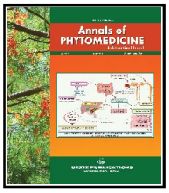
Annals of Phytomedicine 13(1): 988-993, 2024

DOI: http://dx.doi.org/10.54085/ap.2024.13.1.106

Annals of Phytomedicine: An International Journal

http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

An RP-HPLC method development and validation of organic impurities in isotretinoin

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Article Info

Article history

Received 3 May 2024

Revised 20 June 2024

Accepted 21 June 2024

Published Online 30 June 2024

Keywords

Isotretinoin

HPLC

Soft gel capsules

PDA detector

Abstract

A strong oral drug that belongs to the retinoid family, isotretinoin (ISO) is mostly used to treat severe acne. The goal of the current work was to provide analytical techniques, such as HPLC, for ISO estimation in soft gel capsule forms. Its effectiveness in treating acne vulgarism is well-established, but because of its limited therapeutic index and the possibility of side effects, its therapeutic usage has to be precisely quantified. High-performance liquid chromatography has become a dependable method for measuring ISO quantitatively. This study focuses on methods for sample preparation, chromatographic conditions, and detection strategies. The influence of many chromatographic factors on the sensitivity, selectivity, and resolution of ISO peaks is reviewed, including column type, mobile phase composition, and detection wavelength. The present study which employs degassed n-hexane, ethyl acetate, and glacial acetic acid (970:30:0.1) selected as a mobile phase, makes use of HPLC. To accomplish chromatographic separation, the Shimadzu HPLC system with a PDA detector in 385 nm and silica (250 × 4.6 mm, 5 µm) was utilized. Injected at a flow rate of 0.1 ml/min into the column. The drug solution had a 50 µl load volume and an 80 min run period. It was ambient temperature (25°C) in the column. Additionally, to guarantee the dependability and repeatability of the analytical procedure, validation characteristics such as specificity, linearity, accuracy, and precision are clarified. The study also emphasizes how crucial it is to validate methods by ICH criteria to guarantee the accuracy and dependability of analytical results. All things considered, this abstract offers a thorough summary of the analytical techniques for measuring ISO.

1. Introduction

One of the most beneficial vitamin A derivatives that impact all the key elements contributing to the pathophysiology of acne is ISO, an active pharmaceutical ingredient used orally to treat severe forms of acne (Ahmad and Iftekhar, 2023; Gadhimathi *et al.,* 2023). The structure of ISO is shown in Figure 1. It is an isomer of alpha within, a stereoisomer of tretinoin, and a cis-vitamin A acid, a synthetic derivative of vitamin A acid (Akella Anuradha, *et al.,* 2023). It is a retinoid, and it's chemically described as (2Z,4E,6E,8E)-3,7-dimethy l-9-(2,6,6-trimethylcyclohexen-1-yl) nona-2,4,6,8-tetraenoic acid. Isotretinoin is practically insoluble in water and has a low molecular weight (300.44 gm) (Indumathy *et al.,* 2023).13-cis retinoic acid (ISO) is one of the most valuable vitamins. It is still the recommended medication for treating severe, resistant nodular acne, having received approval in 1982 (Nivetha *et al.,* 2023). The only medication that targets every key element contributing to the etiology of acne is (ISO) (Pagade and Sagar, 2023). Strongly lipophilic, isotretinoin is just slightly soluble in oil and nearly insoluble in water. Its molecular formula is $C_{20}H_{28}O_2$, and its molecular weight is 300.44 g/mol. The first-generation drugs, retinoic acids, are still the most effective in treating illness, even though the generations of retinoids are now being studied (Subhamalar *et al.,* 2023).

The literature review (Carla Aiolf and Farid Menaa, 2010; Hsi *et al.,* 2018; Pratik Patel *et al.,* 2011; Wvss Ronald and Bucheli, 1997; Mohit Mahajan *et al.,* 2015; Chinmoy Roy and Jitamanyu Chakrabarty 2013; Nazrul Haq *et al.,* 2023) states that no analytical methodologies have been created in RP-HPLC for isotretinoin capsules.

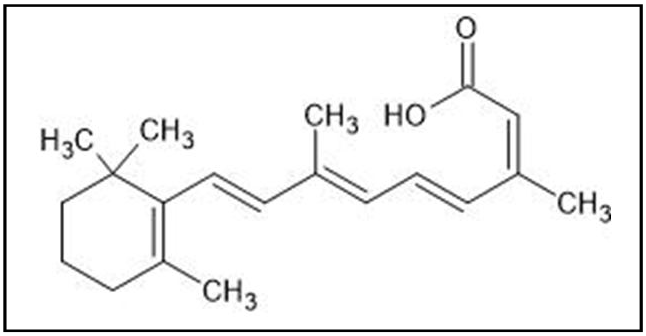

Figure 1: Structure for isotretinoin.

2. Materials and Methods

2.1 Instrumental details

The instruments utilized were Shimadzu high-performance liquid chromatography systems with PDA detectors and analytical balances.

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To accomplish the chromatographic separation, the Shimadzu HPLC system with a PDA detector and silica (250 × 4.6 mm, 5 µm) was utilized. Mix n-hexane, ethyl acetate, and glacial acetic acid (970:30:0.1) made up the mobile phase degassed and injected at a flow rate of 1.0 ml/min onto the column. The drug solution had a 50 µl load volume and an 80 min run period. It was ambient temperature (25°C) in the column.

2.2 Preparation

2.2.1 Mobile phase

Combine glacial acetic acid, n-hexane, and ethyl acetate (970:30:0.1).

2.2.2 System suitability

Measure around 5.0 mg of isotretinoin and 5.0 mg of tretinoin in a 5 ml volumetric flask. After adding the methylene chloride reagent and sonicating for five min, fill the flask to the brim with 5 ml. Once a known concentration of 0.01 mg/ml is reached, transfer 1 ml of this solution into a 100 ml volumetric flask and dilute with diluent.

2.2.3 Standard

Accurately measure and fill a 10 ml volumetric flask with 5 mg of tretinoin. To fill the flask to the top, dissolve the methylene chloride reagent and add it. Pour 5 ml of this mixture into a 50 ml volumetric flask, and then top it out with hexane. Pour 1 ml of the mixture into a 50 ml volumetric flask using a pipette, and then fill it up with n-hexane.

2.2.4 Unspiked sample

Ten soft gel capsules containing 200 mg of isotretinoin were weighed and carefully opened without losing any substance. The contents were transferred to a beaker and washed with 5 ml of methylene chloride reagent per capsule, followed by rinsing with hexanes. Hexanes were used to dilute the mixed washings to volume in a 500 ml volumetric flask. Subsequently, 50 ml of this mixture was moved to a 200 ml volumetric flask and filled with hexane.

2.2.5 Spiked sample

Ten gelatin capsules, weighing a total of 200 mg of isotretinoin, were precisely opened ensuring no loss of substance in a beaker. To transfer the contents, each capsule was washed with 5 ml of methylene chloride reagent and then with hexanes. In a 500 ml volumetric flask, 4 ml of Solution A was added to the washing, and the volume was adjusted with hexanes while stirring. After that, 50 ml of this solution was moved to a 200 ml volumetric flask.

2.2.6 Placebo

1700.0 mg of shell material and 3100.0 mg of placebo medication were weighed in a beaker. After adding 5 ml of the methylene chloride reagent, hexane was used to wash the mixture. After the washing was gathered into a 500 ml volumetric flask, n-hexane was added and the mixture was diluted to volume. Then, 50 ml of this solution was poured into a 200 ml volumetric flask, with n-hexane filling the remaining capacity.

3. Results

3.1 System suitability

Both the system suitability solution and the tretinoin standard solution are ready. To confirm system suitability parameters, six injections of the standard solution and one injection of the system suitability solution were made in the HPLC system. The area response is 398892, with a mean retention time (RT) of 42.029 sec. The standard deviation of RT is 0.014, while the peak width at half height (PW at 50%) is 4024. Furthermore, the total response for the six injections is 1.0 and the (%RSD) for RT is around 0.03.

3.2 Specificity

It denotes its ability to quantify the analyte amidst matrix elements. This is achieved by identifying the analyte, demonstrating specificity through blank and placebo interference, and confirming the analytic's peak purity. The following solutions were made and added to the HPLC equipment to establish specificity: sample solution, spiking sample solution, standard solution, blank, and placebo.

Table 1: Known impurities for sample preparation

Preparation	RT	Area	Peak purity index
Blank	NA	NA	NA
Placebo	NA	NA	NA
Std solution of tretinoin	42.17	376447	0.999987
Spiked sample (tretinoin)	41.95	453309	0.999991
Spiked sample (isotretinoin)	30.49	18984403	0.999985

3.3 Linearity and range

3.3.1 Linearity

The concentration of the analyte in samples directly correlates with the method's ability to generate accurate results within a certain range.

3.3.2 Range

The range of analyte concentrations, in cases where the precision, accuracy, and linearity of the analytical approach are adequate, spans from 50.0% to 150.0% of the specified limit for the unknown impurity (0%). Linearity was established at concentration levels of 50.0%, 75.0%, 100.0%, 125.0%, and 150.0%, with intercept and correlation coefficients noted. To calculate the slope, record the average area for each concentration level. Following that, plot the analyte peak concentration on the X-axis of the graph and the area response on the Y-axis.

3.4 Accuracy

Accuracy is determined by how closely the test results generated by a procedure match the actual value. Per cent recovery, calculated from known, added quantities of analyte, is a common indicator of

accuracy. Using concentrations of 50.0%, 100.0%, and 150.0%, accuracy was assessed for tretinoin standard and spiked sample solutions, prepared at these concentrations relative to the specified limit of the unknown impurity (1.0%). For every solution, three injections were made; the average area obtained for each concentration was used to compute the percentage of recovery.

Table 2: Linearity result

Level	Wt. tretinoin taken in mg	Diluted to vol. with blank (ml)	Vol. of stock soln taken in ml	Diluted with vol. with blank	Vol. of stock soln taken in ml	Diluted with vol. with blank	Conc. in %
50					0.5	50	50
75					1.5	100	75
100	5.0	10	5	50	1.0	50	100
125					2.5	100	125
150					1.5	50	150

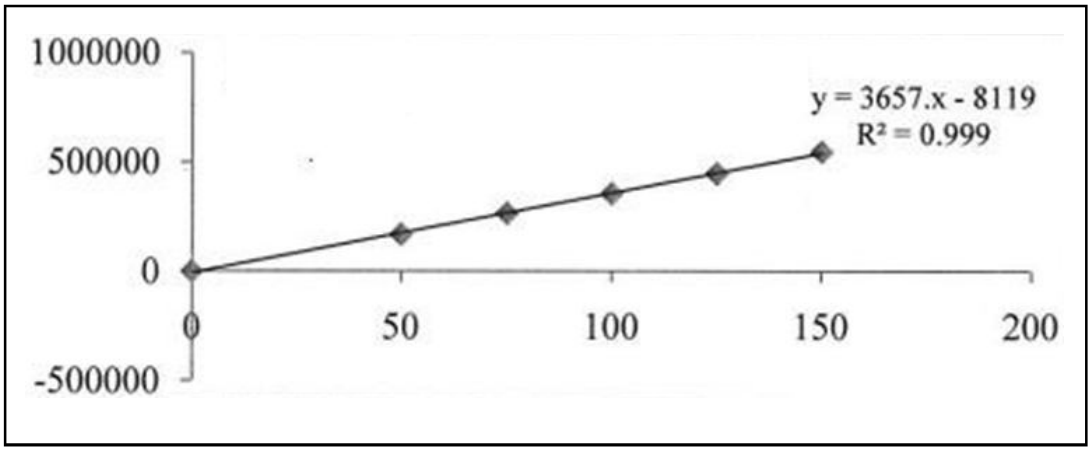

Figure 2: Linearity curve for isotretinoin.

Table 3: Accuracy result of isotretinoin

Conc.	Amt. of isotretinoin added in ppm	Amt. of isotretinoin recovered in ppm	Recovery	Avg in%	RSD in %
50.0%	0.516	0.539226	104.5011	104.8	0.2
	0.516	0.541304	104.9038		
	0.516	0.541235	104.8905		
100.0%	1.032	1.023518	99.1781	98.2	1.2
	1.032	1.000448	96.9426		
	1.032	1.017202	98.5660		
150.0%	1.548	1.471694	95.0706	96.2	1.0
	1.548	1.498668	96.8131		
	1.548	1.494915	96.5707		
		Average		**99.7**	**0.8**

3.5 Precision

The degree of agreement between individual test findings acquired when an analytical procedure is used repeatedly on homogenous samples is referred to as precision. The assessment of impurities in six aliquots of a homogeneous sample is utilized to evaluate the precision of the analytical method.

3.5.1 System precision

Six duplicate injections of the tretinoin working standard solution were carried out to evaluate the accuracy of the system. For the peak region, the percent relative standard deviation (%RSD) was computed. Additionally, it was observed that the retention time for the isotretinoin capsules was 42.0, and the %RSD for the peak area of the isotretinoin capsules was 398892.

3.5.2 Method precision

The accuracy and repeatability of the method can be demonstrated by analyzing the percentage of unknown impurities and the

percentage of total impurities in six duplicate samples of isotretinoin capsules. By analyzing six identical samples of isotretinoin capsules, the %RSD and impurity levels derived from the six replicate preparations showcase the method's accuracy and repeatability.

3.5.3 Intermediate precision

To ensure intermediate accuracy, six duplicate samples of isotretinoin capsules were analyzed in a laboratory setting by multiple analysts on different days. The computed percentage relative standard deviation (%RSD) of the findings was then compared to assess the consistency and reliability of the results obtained across different analysts and days.

The accuracy and repeatability of the procedure are demonstrated by analyzing the percentage of contaminants from six duplicate preparations and comparing the percentage of relative standard deviation (%RSD) values acquired by analysts 1 and 2 on two separate days utilizing two different HPLC equipment and columns.

Table 4: Unknown impurities for sample preparation

Column details	Max. unknown impurities (%)	
	Method precision	Intermediate precision
	Silica, (250 × 4.6 mm, 5 µm) column in Shimadzu HPLC	Silica, (250 × 4.6 mm, 5 µm) column in Agilent HPLC
Sample details	**Tretinoin**	**Isotretinoin**
1	0.0349	0.0287
2	0.0319	0.0286
3	0.0349	0.0285
4	0.0342	0.0288
5	0.0335	0.0286
6	0.0338	0.0284
Avg	**0.0339**	**0.0286**
%RSD	**3.2**	**0.3**
Overall % RSD	**9.0**	
Confidence limit	**0.0**	**0.0**

Table 5: Total impurities for sample preparation

Instrument details	Total impurities (%)	
	Method precision	Intermediate precision
	Shimadzu HPLC	Agilent HPLC
Column details	**Silica, (250 X 4.6 mm, 5 µm) column in Shimadzu HPLC**	**Silica, (250 X 4.6 mm, 5 µm) column in Agilent HPLC**
1	0.0563	0.0498
2	0.0549	0.0468
3	0.0580	0.0464
4	0.0577	0.0469
5	0.0577	0.0490
6	0.0568	0.0469
Avg	0.0569	0.0476
%RSD	1.9	2.9
Overall % RSD	**9.4**	
Confidence limit	**0.0**	**0.0**

3.6 Stability

For a maximum of 48 h, the standard and sample solutions are injected every 12 h to show the stability of the solution. The relative standard deviation (RSD) of the area of the solutions obtained at various time intervals confirms that the standard and sample solutions are stable for a maximum of 48 h at room temperature.

Table 6: Maximum unknown impurities for sample

Time interval	Standard	Sample
		% Max. unknown impurities
Initial	376447	13807
24th h	371669	14154
48th h	388880	13877
Average	378999	13946
Std dev	8884.699	183.5020
RSD (%)	2.3	1.3

4. Discussion

High-performance liquid chromatography (HPLC) has proven to be a valuable analytical tool for the quantitative determination of isotretinoin, particularly in the context of relative substance analysis. The precise quantification of isotretinoin is essential due to its narrow therapeutic window and potential for adverse effects. The comprehensive examination of various chromatographic parameters, sample preparation techniques, and validation parameters underscores the importance of method development and validation in ensuring accurate and reliable results. The optimization of chromatographic conditions, including the selection of appropriate columns, mobile phase composition, and detection wavelength, is crucial for achieving satisfactory resolution and sensitivity for isotretinoin peaks amidst complex matrices. Additionally, isolating isotretinoin from interfering substances.

5. Conclusion

The Validation guidelines, including specificity, linearity, accuracy, precision, and robustness, are imperative for ensuring the method's reliability. By validating the analytical method according to international standards, analysts can confidently apply the developed HPLC method for routine quality control and pharmacokinetic studies of Isotretinoin-containing formulations. In conclusion, the continued advancement and refinement of HPLC methodologies for the analysis of Isotretinoin contribute significantly to pharmaceutical research, clinical practice, and regulatory compliance. By employing optimized analytical methods with rigorous validation, researchers and analysts can effectively monitor the quality, safety, and efficacy of isotretinoin formulations, thereby enhancing patient care and therapeutic outcomes in dermatological practice.

Acknowledgments

The author thanks the management and staff, Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), and the company for lending us the raw materials and workspace necessary to complete this work.

Conflicts of interest

The authors declare no conflict of interest relevant to this article.

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Citation **R. Gandhimathi and S. Harishini (2024). An RP-HPLC method development and validation of organic impurities in isotretinoin. Ann. Phytomed., 13(1):988-993. http://dx.doi.org/10.54085/ap.2024.13.1.106.**