

## IDENTIFICATION, SEPARATION, AND CHARACTERIZATION OF DEGRADATION PRODUCTS OF TRIAMCINOLONE HEXACETONIDE USING LC AND LC-MS/MS

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Received: 15 Dec 2023, Revised and Accepted: 10 Feb 2024

### ABSTRACT

**Objective:** The study aimed to separate the forced degradation products of Triamcinolone hexacetonide using HPLC and characterize the degradation product by LC-MS/MS fragmentation pattern.

**Methods:** Triamcinolone hexacetonide (THA) and its primary degradation products were identified using a liquid chromatography-mass spectrometry/Mass spectrometry (LC-MS/MS) approach. The degradation study was based on in-depth stress testing with acid, base, peroxide, heat, and light. A Zorbax SB C18 column and a greener mobile phase composed of methanol and 10 mmol ammonium acetate buffer in water at pH 3 were employed to accomplish separation and quantitation at a flow rate of 0.7 ml/min in an isocratic mode with a 239 nm detection wavelength.

**Results:** A major degradation product of the drug was obtained in acidic and alkaline stress conditions. The drug was found to be stable for all other stress conditions. The LC-MS/MS analysis results of the active pharmaceutical ingredient and resulting product after degradation were interpreted to identify the novel degradation product and fragments. The developed method was validated as per International Council for Harmonization (ICH) guidelines. The square root of the correlation coefficients, which indicated linearity for THA in 50 to 150 % of the workload, was 0.99. Method Precision assay was performed on six different preparations, percentage relative standard deviation (% RSD) of assay value is 0.17 % and system precision is 0.30 %. In accuracy, overall % RSD of 50 %, 100 %, and 150 % in triplicate is 0.95.

**Conclusion:** It is concluded that the drug is stable to all other stress conditions except for acidic and alkaline stress conditions and generates a novel degradation product. The developed LC (Liquid chromatography) method separates and identifies the degradation product.

**Keywords:** Stress degradation product, LC-MS/MS, Triamcinolone hexacetonide, Validation

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### INTRODUCTION

Triamcinolone hexacetonide (THA) is a synthetic glucocorticoid analog in the form of powder or suspension for injection (KENACORT HEXA) utilized to treat inflammatory joint diseases such as juvenile inflammatory arthritis (JIA), Rheumatoid arthritis (RA), and osteoarthritis (OA) by intraarticular injection. It interacts with glucocorticoid receptor response elements on deoxyribonucleic acid (DNA) after binding to certain cytosolic glucocorticoid receptors in the target cell, changing the expression of related genes [1-9].

A stress study is necessary to establish degradation behavior as well as the shelf life and storage conditions of a drug. The developed method for stress study needs to be validated by following ICH guidelines for universal acceptance [10]. The HPLC methods available on THA include simultaneous estimation and pharmacokinetic and pharmacodynamic studies [11-16] but provide no information about THA degradation. There is not currently a stability-indicating method (SIM) for THA.

As the THA is available as it is in powder form or suspension form without other excipients, this stress study can be directly correlated with the available formulations. Hence, the development and validation of an LC SIM for THA was the objective of this investigation. After analyzing the results of the series of stress tests, a novel degradation product was obtained and characterized by LC-MS/MS fragmentation results.

The greening of RP-HPLC procedures has garnered much interest in the analytical community, which is looking for new solutions to replace polluting analytical methods with cleaner ones, due to the health and environmental concerns of organic solvents widely employed in RP-HPLC. In the 2000s, green chemistry gave rise to green analytical chemistry (GAC), which has since acquired popularity among academics. The idea behind it is to eliminate or

reduce harmful substances from analytical processes to promote environmental and human health without sacrificing method performance. In this study, we tried to use environmentally low-risk green solvents as much as possible [17].

### MATERIALS AND METHODS

#### Chemicals

HPLC grade methanol, Water (H<sub>2</sub>O), and Acetic acid (CH<sub>3</sub>COOH) were obtained from Advent Lab. (Pune, India). Analytical grade ammonium chloride, hydrochloric acid, hydrogen peroxide, and sodium hydroxide were obtained from Advent Lab (Pune, India). 1 mol hydrochloric acid and 0.5 mol sodium hydroxide solutions were prepared. THA was obtained from Avik Pharmaceutical Limited, Gujrat as a gift sample for this research study. Waters HPLC Model 2695 and dual UV detector 2487 were used to develop and validate the method. For peak purity determination, Waters HPLC Model 2695 and PDA detector 2996 were used.

#### HPLC-UV analysis

For chromatography, a Waters HPLC Model 2695 and dual UV detector 2487 were used for method development and validation. For peak purity determination Waters HPLC Model 2695 and PDA detector 2996 were used. A Zorbax SB C18 column (250 × 4.6 mm, 5 μm particles) was used as the stationary phase. The flow rate employed was 0.7 ml/min. UV absorbance wavelength was set at 239 nm. The isocratic Mobile phase was composed of methanol and 10 mmol ammonium acetate buffer in water at pH 3 with Glacial acetic acid (90:10). Injection volume was 20 μl. Chromatograms were obtained and analyzed with Empower2 software.

#### Stress testing

The stress testing was carried out as per ICH guidelines ICH Q1A (R2) [18]. 1000 μg/ml solutions of THA in diluent (same as mobile

phase) were used for stress testing. The stress conditions were optimized to obtain a stressed product target of 6–15 %. 1 mol HCl and 0.5 mol NaOH solutions were employed to obtain acid and base-mediated hydrolysis. A 3 % H<sub>2</sub>O<sub>2</sub> solution was used to stimulate peroxide-mediated oxidation. Light and thermal stresses were also investigated. The final concentration for stress testing was 100 µg/ml.

#### LC-MS analysis

Mass analysis was carried out on an Agilent 6530 Q-TOF system with an electrospray ionization (ESI) source. Scanning range for masses was m/z 50–600, gas flow to 10 l/min, gas temperature to 350 °C, and voltage 3.5 kV. Mass spectra were analyzed with Agilent Mass Hunter software. The developed HPLC method was MS compatible; hence the same method was used for MS analysis. Mass spectra are included in supplementary data.

#### Sample preparation

API sample was solubilized in methanol and 10 mmol ammonium acetate solution in water buffered at pH 3 with acetic acid (9:1) to prepare a 10 µg/ml solution.

#### Method validation

Accuracy, precision (including both repeatability and intermediate precision), specificity, linearity, detection, and quantification limits (LOD and LOQ) were assessed. ICH Q2 (R1) guidelines were followed to validate the method [19-21].

#### Accuracy

Freshly prepared THA solutions in concentrations of 50 % 100 % and 150 % of the 10-µg/ml workload were analyzed. Two consecutive runs for each load were considered to estimate recovery (n=6).

#### Precision

Six consecutive injections of 100 % and the workload for THA in methanol buffer (9:1) were analyzed for assessment of the precision of the method (n=6).

#### Specificity

The PDA detector was used to determine specificity by estimation of peak purity which was found to be fair.

#### Linearity, range, LOD, and LOQ

Linearity was determined for THA at 50, 80, 100, 120 and 150 % of the workload (n=5). For LOD and LOQ determination, the

concentrations 10 times less than workload concentration using a signal-to-noise (s/n) ratio were analyzed. (s/n more than 10:1 as well as area reproducibility for LOQ; and more than 3:1 for LOD).

#### Robustness

The influence of flow rate on the peak area, theoretical plates, and tailing factor of the standard was tested by using a flow rate variation of 0.6, 0.7, and 0.8 ml/min (n=3).

#### Stability of solution

The six replicate injections of standard untreated THA solution were run and the same solution was injected after 24 and 48 h in duplicate to determine the stability of the solution. The % RSD was found to be 1.13, which is acceptable (n=12).

### RESULTS AND DISCUSSION

#### Optimization of chromatographic conditions

The starting point of the optimization was the THA-related substances method of the European Pharmacopoeia (monograph 01/2005:0867). In the Pharmacopoeia method, the retention time for THA is 12 min with a flow rate of 2.0 ml/min without the use of a buffer. But to separate possible degradation products, it is required the pH of the mobile phase should remain constant and separation should be achieved earlier than 12 min to reduce the run time. Therefore, retention was reduced by shifting the isocratic flow from 75:25 (methanol: water) to 90:10 (methanol: buffer). Though 20 µg/ml and 25 µg/ml solutions are used in European Pharmacopoeia, we selected 10 µg/ml concentration as a good area, and symmetry of peak was obtained on the HPLC system. In other existing methods acetonitrile (ACN) was used as the component of the mobile phase [15, 16], which is carcinogenic and more toxic as compared to alcohols [17]; hence we had completely avoided the use of ACN.

#### Stress testing results

Major degradation occurred in alkaline hydrolysis. The base degradation obtained was 14.91 % and the peak was obtained at 4.53 min. The same degradation product was obtained in acid degradation but to a lower extent (4.82 %). The peak of standard THA was obtained at 7.80 min. The drug was found to be more labile to alkaline stress conditions compared to acidic stress conditions as the THA gives immediate degradation product with alkali (2 min) of low concentration (0.5 mol). No degradation occurred under oxidative, thermal, and photolytic stress conditions. The chromatograms of base and acid degradation of THA are shown in fig. 1, and fig. 2, respectively.

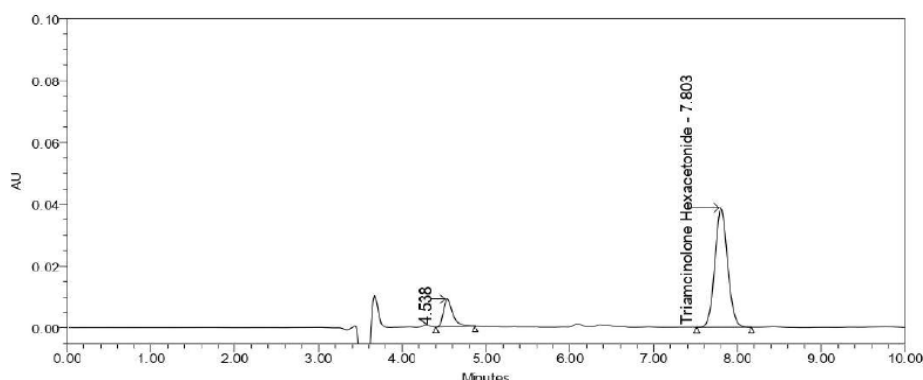


Fig. 1: Chromatogram of base degradation product and THA

#### THA lc-ms/ms analysis

The molecular ion peak for Triamcinolone hexacetonide was obtained at m/z ratio 533.29. The sodium (m/z 555.27) and potassium (m/z 571.24) adducts were observed in the LC-MS/MS chromatogram. Similar fragments were obtained for the drug Triamcinolone acetonide, which is an analogue of THA [22]. The first fragment of the drug was obtained at an m/z ratio of 513.28,

which is likely to be formed by the removal of the fluorine atom from the 7<sup>th</sup> carbon with the acquisition of a plus charge. Further fragment of THA was obtained at an m/z ratio of 495.27 due to the loss of the hydroxyl group and hydrogens from the 7<sup>th</sup>, 8<sup>th</sup>, and 11<sup>th</sup> positions of the drug to form 2 water molecules. The next fragment formed due to the removal of the isobutane group from the 23<sup>rd</sup> carbon (m/z ratio 439.21). Further formaldehyde removal resulted in another fragment (m/z ratio 411.21), leaving the

methoxy group at 21<sup>st</sup> carbon. This methyl group was removed as methane, leaving the hydroxyl group at 21<sup>st</sup> carbon (m/z ratio 397.20). The next fragment was observed to be formed by the removal of formic acid from 20<sup>th</sup> carbon (m/z ratio 339.19). Removal of ethane molecule resulted in fragment with m/z ratio 321.14. The dioxolane ring then hydrolyzed leaving methyl and hydroxyl groups at 17<sup>th</sup> carbon (m/z ratio 293.15). The D ring of the steroid nucleus hydrolyzed further to form a fragment with an m/z ratio of 253.16. Removal of ethane gas resulted in an m/z

ratio of 225.12 fragment. The C ring of the drug hydrolyzed further resulting in a 3-oxo fragment (m/z ratio 147.08). Ethylene gas was removed by the breakdown of the B ring of the drug further to form a fragment with an m/z ratio of 121.06. The A ring was also found hydrolyzed, forming a fragment (m/z ratio 99.08). The last fragment (m/z ratio 57.03) observed in the chromatogram resulted from removal of the propane molecule. The fragments obtained through lc-ms/ms analysis are depicted in fig. 5 and the chromatogram is given in fig. 6.

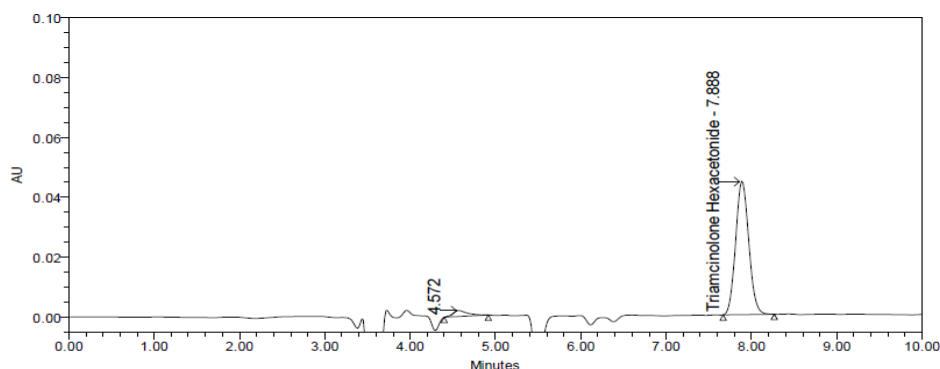


Fig. 2: Acid degradation product and THA

### DP-I lc-ms/ms analysis

The DP-I was formed during the alkali hydrolysis of the drug. The molecular ion peak of DP-I was found at an m/z value of 515.30, which was exactly 17.99 amu less than the drug Triamcinolone hexacetone (m/z = 533.29). It is implied that the 7-fluoro atom is removed by an alkali as Sodium fluoride and is replaced by an H atom (-18.99(F)+1.00(H) = 17.99) to form the DP-I. Similar degradation with fluoride removal was obtained for the

Triamcinolone acetonide drug (analog of THA) in a previous study [23].

It is observed that DP-I followed the same fragmentation pattern as that of the drug. The sodium (m/z 537.28) and potassium (m/z 553.25) adducts were observed in the LC-MS/MS chromatogram. Further fragmentation pattern of the degradation product DP-I was found to be the same as that of the drug. The fragmentation pattern for DP-I is depicted in fig. 3, and the chromatogram is given in fig. 4.

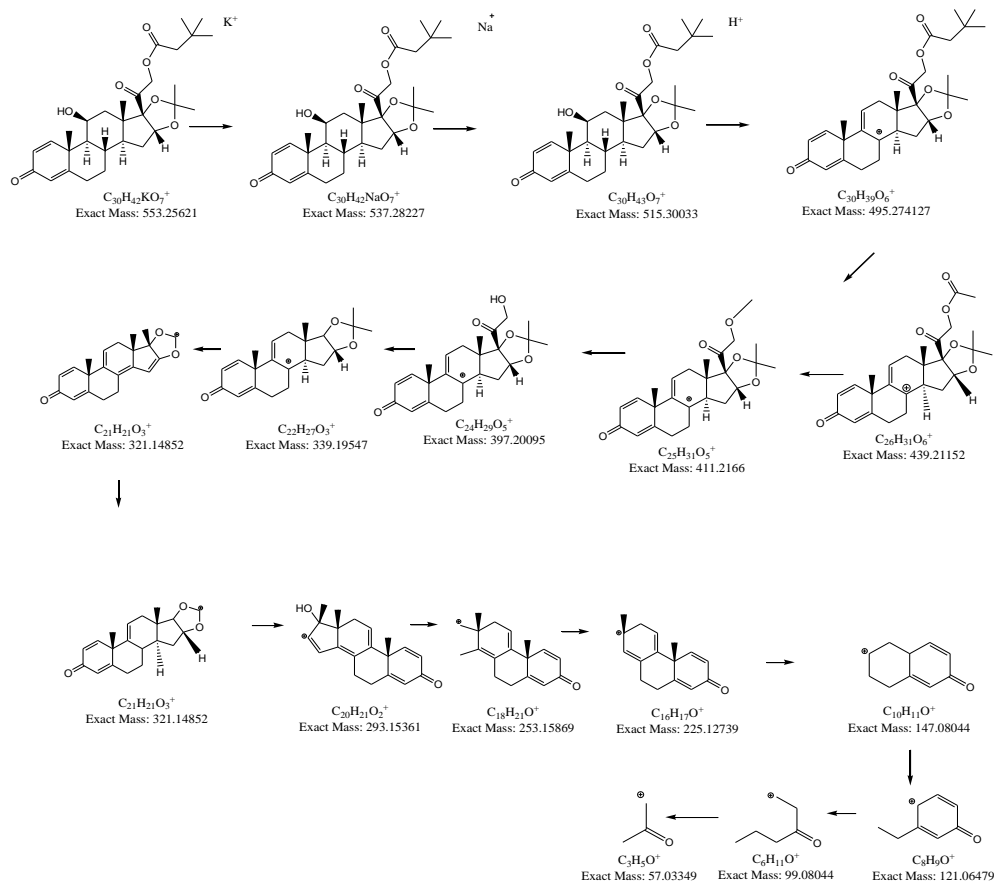


Fig. 3: Base degradation product fragmentation of THA by LC-MS/MS

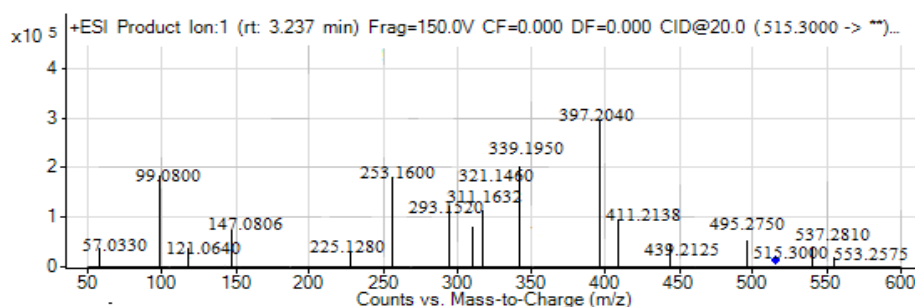


Fig. 4: LC-MS/MS chromatogram of (DP I) base degradation product of THA

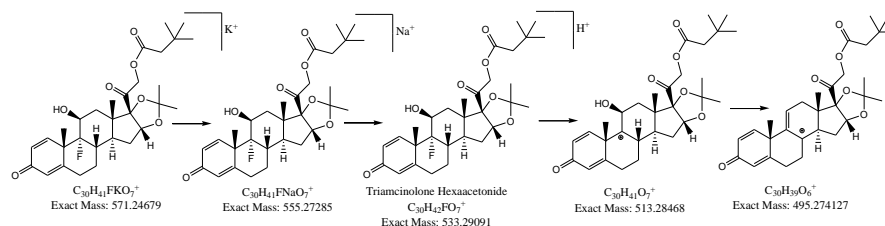


Fig. 5: Fragmentation of THA standard by LC-MS/MS

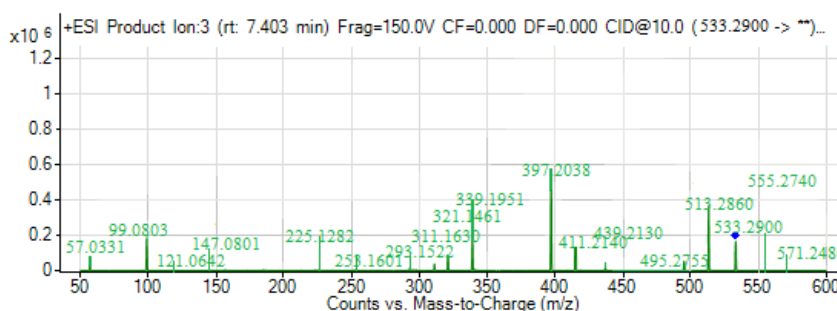


Fig. 6: LC-MS/MS chromatogram of standard THA

**Method validation results**

The method validation results are summarized in table 1 and further discussed in the following paragraphs. The overall results of % RSD,

number of observations (n), and mean±SD values are shown in table 1. The values for linearity, LOD, and LOQ were calculated from the areas of standard THA linearity concentrations in the range from 5–12 µg/ml; the results obtained are summarized in table 2.

**Table 1: Method validation parameters and results**

Parameter	Description of test	% RSD	Results % (mean±SD, n)
Accuracy	Mean % recovery of the standard THA	0.95	100.18±0.90, n=6
Precision	Interday	0.17	99.18±0.17, n=6
	Intraday	0.18	99.82±0.19, n=6
	System suitability	0.30	99.94±0.30, n=5
Stability of solution	% RSD for over a range of 48 h	1.13	100 %±1.13, n=12
Robustness	% RSD for flow variation	0.57	100 %±0.57, n=3

(n-number of observations, % RSD-percentage relative standard deviation, LOD-limit of detection, LOQ-limit of quantification, THA-Triamcinolone hexaacetonide), The results are shown as mean±SD and number of observations (n).

**Table 2: Linearity, LOD, and LOQ results**

Parameter	Description of test	Results by calculation
Linearity	Coefficient of correlation	0.99, n=5
LOD	Limit of detection	2.21 µg/ml
LOQ	Limit of quantitation	6.67 µg/ml

(n-number of observations, LOD-limit of detection, LOQ-limit of quantification), the results are obtained by statistical calculations from linearity data.

**Accuracy**

Recovery of THA from a freshly prepared solution was determined six consecutive times. The mean recovery was 100.18 %±0.90 (RSD 0.95 %, n = 6) for THA.

**Precision**

% RSD of THA for system suitability precision carried out on five consecutive injections (n= 5) is 0.30. The % RSD of THA for intraday

precision carried out on six consecutive injections (n= 6) is 0.18, and the % RSD of THA for intermediate precision is 0.30 (n= 6).

**Specificity**

For specificity determination, the THA sample was run on a PDA detector and no flag was obtained for peak purity. Though the peak height is low for THA peak, it is evident from the peak area of around 5 lakhs, chromatogram for 1 µg/ml solution of THA, and s/n ratio that the method is specific. Both chromatograms are given in fig. 7.

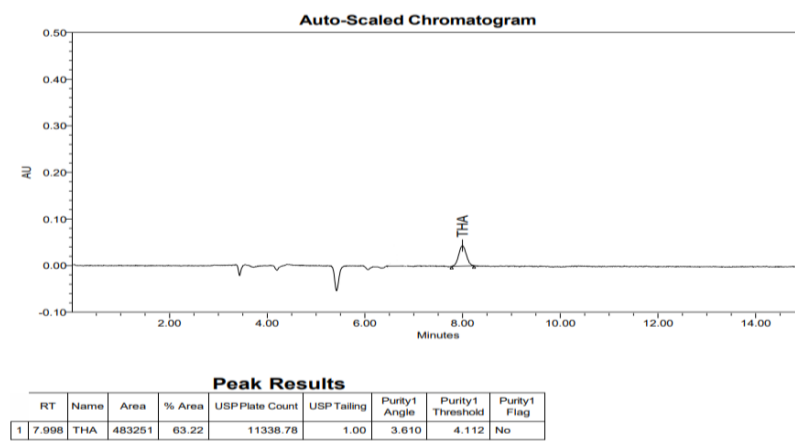


Fig. 7A: Peak purity chromatogram

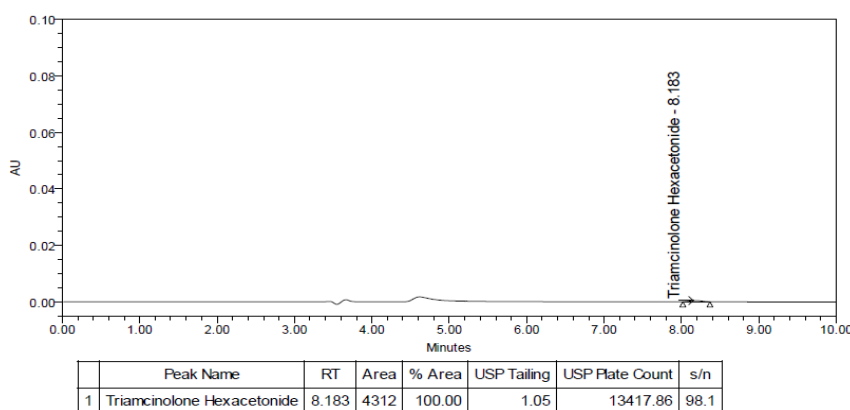


Fig. 7B: Chromatogram for low concentration (1 µg/ml) of THA

**Linearity, range, LOD, and LOQ**

The linearity of THA for the workload of 50, 80, 100, 120 and, and 150 % was within the range with R<sup>2</sup>= 0.9981 (n=5). Fig. 8 presents

the calibration curve. LOD and LOQ were found to be 2.21 µg/ml and 6.67 µg/ml, respectively by calculations derived from linearity data, given as per ICH guidelines [19].

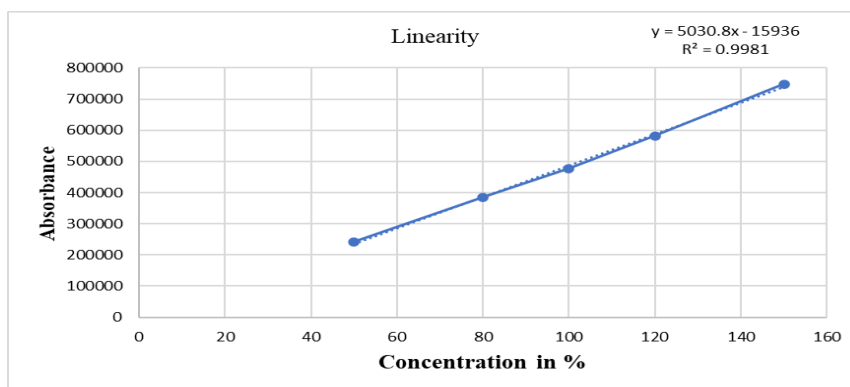


Fig. 8: Chart of linearity and coefficient of correlation. The coefficient of correlation for linearity was 0.9981 (n=5)

## Robustness

Robustness concerning the flow rate was performed by three injections each of standard THA and test THA at 0.6 ml/min, 0.7 ml/min, and 0.8 ml/min (n=3). The percentage RSD was 0.57. The pH deviations were not studied as buffer composition in the mobile phase was only 10 % which is negligible to assess pH deviation.

## CONCLUSION

An HPLC method was developed and validated using a green mobile phase. THA was found to be unaffected at photo, peroxide, and thermal stress conditions except for acid and base degradation giving the same degradation product. The degradation product was identified using LC-MS/MS. The novel findings from this work are the degradation product and fragments of a degradation product. The described method can be used in practice for triamcinolone hexacetonide powder for injection.

## ACKNOWLEDGMENT

The author would like to extend heartfelt thanks to Savitribai Phule Pune University and Sinhgad Institute of Pharmacy, Narhe, Pune, Maharashtra, India for providing instrumental facilities and Food Testing Laboratory of Bharati Vidyapeeth Deemed University, Pune, Maharashtra, India for helpful guidance. The authors are also thankful to Avik Pharmaceuticals Pvt. Ltd. Gujrat for providing the API Triamcinolone hexacetonide as a gift sample for this research.

## FUNDING

Nil

## ABBREVIATIONS

DNA: Deoxyribonucleic acid, DP: Degradation Product, GAC: Green Analytical Chemistry, ICH: International Council on Harmonization, JIA: Juvenile Inflammatory Arthritis, OA: Osteoarthritis, PDA: Photodiode Array, RA: Rheumatoid arthritis, SIM: Stability Indicating Method, STD: Standard, THA: Triamcinolone hexacetonide.

## AUTHORS CONTRIBUTIONS

All authors are contributed equally

## CONFLICT OF INTERESTS

Authors declare no conflict of interest

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