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Δ14-STEROIDAL IMPURITY: PREPARATION, CHARACTERIZATION AND EVALUATION, AS WELL DEVELOPMENT OF NEW HPLC METHOD IN DRUG SUBSTANCE LIKE TRIAMCINOLONE HEXACETONIDE

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ABSTRACT

The evaluation of pharmaceutical raw materials and finished products for impurities and degradation product is vital part of the drug development and testing procedure. Furthermore, separation of any drug-related impurity that is present at a concentration of less than 0.05% of that of the active pharmaceutical ingredient (API) is also critical part of process development and process validation. Present innovation includes identification by LC-MS, of overlapping unknown impurity, which is a carry forwarded impurity from 16α, 17α, 21-Trihydroxy-9, 11-Oxidopregna-1, 4-diene-3, 20-dione (5TR) (Key Starting Material), with that of Impurity-B and Δ^{14}-Triamcinolone Hexacetonide (the most probable impurity in the process of Triamcinolone Acetonide to Triamcinolone Hexacetonide) of Triamcinolone Hexacetonide in pharmacopeial HPLC method of analysis. It's identification, characterization as a Δ^{14} steroidal impurity, synthesis, assessment, and New (In-house)superior HPLC method development to separate it from API as well as other impurities including impurity B. Moreover, the synthesized Δ^{14} steroidal impurity converted to Acetonide and Hexacetonide family successfully.

KEYWORDS

Impurity, LC-MS, EP-9.0, Corticosteroid, Triamcinolone Hexacetonide and Separation.

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INTRODUCTION

Triamcinolone Hexacetonide[1-3] is a very good fat-soluble glucocorticoid, which is used as a drug for the treatment of inflammatory practices in joints (for example, arthritis). Triamcinolone Hexacetonide is an anti-inflammatory drug from the group of glucocorticoids used to treat rheumatic infections. It is administered intra-articularly[4]. It is a pro drug of Triamcinolone Acetonide, which is

activated by hydrolysis. Triamcinolone Hexacetonide is formulated for parenteral administration via intra-articular, intralesional or sublesional routes. It is marketed under the brand ARISTOSPAN® and is available as an aqueous suspension of 5mg/mL or 20mg/mL micronized Triamcinolone Hexacetonide. (Brand Name: Aristospan: USA, Lederspan: GB, Hexatrione Longue duree: F, Lederlon5/20 (Lederle): D)* U.S. Patent No.3457348 described methods of preparing Triamcinolone Hexacetonide. The anti-inflammatory properties of Triamcinolone Hexacetonide in rabbits are disclosed in Hunneyball, Agents Actions, 11:490 (1981) and early clinical studies are disclosed in Bilka, Minnesota Med., 50: 483 (1967) and Layman and Peterson, Id. at 669.

In present study, we have gone through the purification of Triamcinolone Hexacetonide (TMHA) (I). In some experiments, Impurity-B [III] was reduced below European pharmacopeia limit but in some experiments it was not reduced, highlighting the chances of overlapping of other impurity with Impurity-B (RRT 0.79). Afterwards, LC-MS analysis (Figure No.2) of highly purified Triamcinolone Hexacetonide (TMHA) having 0.79 RRT impurity has been performed, and for our surprise, molecular ion peak corresponding to Impurity B (Molecular Mass: 518.61g/mole) was absent, but actual molecular mass 531.08 m/z (M+1) was observed, justifying the presence of Δ^{14} Impurity (II)[5]. Advance deep studies confirmed the generation of Δ^{14} impurity during the synthesis of 9, 11β-Epoxide Triamcinolone (5TR), which is key starting material and promote carry forward to Triamcinolone family. The same Δ^{14} Impurity (II) of Triamcinolone Hexacetonide was then, synthesized, characterized, and confirmed in laboratory. Likewise, it has been confirmed by spike study along with all other impurities in HPLC of Triamcinolone Hexacetonide (Figure No.3). Additionally, New (In-house) qualitative HPLC technique has been established to get split-up of Δ^{14} Triamcinolone Hexacetonide impurity from Impurity-B besides API (Triamcinolone Hexacetonide).

MATERIAL AND METHODS

Synthesis

Chemicals and reagents

All analytical chemicals and reagents used were of AR grade.

Procedure

Preparation of Δ^{14}-Impurity from 1, 4, 9, (11), 16-Pregnatetraene-3, 20-Dione-21-Acetate (3TR) (IV) to Δ^{14}-Impurity of 16α, 17α, 21-Trihydroxy-9, 11-Oxidopregna-1, 4-diene-3, 20-dione (5TR), followed by Δ^{14}-Impurity of Triamcinolone Acetonide and Δ^{14}-impurity of Triamcinolone Hexacetonide [II], subsequently, have been described. Route of synthesis of Δ^{14}-Triamcinolone Hexacetonide is illustrated in synthetic scheme in Figure No.1.

Bromo-3TR (V)

100g 1, 4, 9, (11), 16-Pregnatetraene-3, 20-Dione-21-Acetate (3TR) (IV) was dissolved in a mixture of 1 liter Acetone and 130ml Demineralized water. Then, reaction mass was cooled to -25°C. 10% aq. perchloric acid was added to it and stirred for 10 min. Then, 51g 1, 3-Dibromo-5, 5-dimethyl hydantoin (DDH) was added lot wise within 25-30 min. Now, it was stirred for 1.5 hr. Reaction completion was confirmed by TLC (Thin Layer Chromatography). Reaction mass, then, added with aqueous sodium bisulfite solution via slow addition pattern. Resulting mass was poured in 1500ml ice cold water. 10% aq. Sodium carbonate solution was added to it upto pH 6.5-7.0. Heterogeneous mass obtained thus was filtered to get 431g wet cake.

Δ^{14}-Bromohydrin (VI)

100g bromo-3TR (Wet Cake) (V), 2 liter Acetic acid, and 3 liter Acetone were mixed. The mixture was chilled to -25°C and stirred for 10 mins at -25°C. Then 10% aqueous potassium permanganate ($KMnO_4$) solution was added to it and stirred for 30 minutes. Reaction completion was confirmed by TLC. 25% Sodium bisulfite solution was added to terminate the reaction and temperature was increased upto 25°C. Reaction mass was poured in ice cold water and extracted twice with methylene dichloride. Consequently, methylene dichloride layer was washed with water thrice followed by

complete solvent distillation and degassing. Dry solid, thus obtained, weighed 88.45g.

Δ^{14}-5TR (VII)

74.8 g Δ^{14}-Bromohydrin (VI), 1122ml methanol, and 374ml methylene dichloride were mixed. This homogenous mixture was cooled -5 to -10°C and stirred for 60 min at the same temperature. Then, 207ml, 10% aqueous potassium carbonate solution was added by slow addition pattern. Reaction progress was monitored by TLC. After completion of the reaction, pH adjusted to neutral by using acetic acid. The mixture was subjected for solvent distillation and distilled out upto formation of thick viscous mass, which was further portioned between water methylene dichloride systems and separated. So obtained, aqueous layer, was extracted twice more with methylene dichloride. All three methylene dichloride layers were combined and left overnight to get precipitated solids. The heterogeneous mass, so obtained, was filtered to get 46.86g wet cake, which was further dried to get 21.90g dry solid in 38.9% yield. IR (KBr): -OH str. (3398cm^{-1}), C=O ketone (1717cm^{-1}), C=C Str. (1666cm^{-1}), C=O ring str. (1620cm^{-1}), C-C ring str. (1378cm^{-1}), C-O str. (1054cm^{-1}).^{1}HNMR (δ, DMSO): 6.688-6.662 (1H, d, -CH=CH-C=O), 6.146-6.141 (1H, d, -CH=CH-C=O), 6.062 (1H, s, CH=CH-C=O), 5.309 (1H, s,-C=CH-CH-OH), 5.295-5.281 (1H, d,-C=CH-CH-OH), 5.225 (1H, s,-C=CH-CH-OH), 2.732-2.658 (1H,t, -CH_2-OH). ESI-MS (m/z): 373.2 (M+1).

Δ^{14}-Triamcinolone Acetonide impurity (VIII) (Impurity B as per EP-9.0)

8g Δ^{14}-5TR (VII) was taken in 240ml acetone, cooled the mass, and stirred for 10 min. The mixture was added with HF (70%) and stirred for 30 minutes. Then, temperature was raised upto 5°-10°C. First reaction (Acetonide formation) completion was confirmed by TLC. Then add extra HF (70%) and temperature was raised upto 25-30°C and maintained for 2.5 hr. Second reaction (Epoxy ring opening) completion was confirmed by TLC. After that, the reaction mass was added with ice, stirred and pH adjusted to neutral using sodium carbonate solution. Then filter the mass to get crude solid. Crude solid, so obtained, was purified using Methanol and MDC to give 1.8 g the Δ^{14}-Triamcinolone Acetonide impurity. IR (KBr): -OH str. (3441cm-1), C=O ketone (1731cm-1), C=C Str. (1665cm-1), C=O ring str. (1625cm-1), C-C ring str. (1379cm-1), C-O str. (1069cm-1). 1HNMR (δ, DMSO): 7.123-7.104 (1H, d, -CH=CH-C=O), 6.354-6.333 (1H, d, -CH=CH-C=O), 6.152 (1H, s, CH=CH-C=O), 5.410 (1H, d, -CH-OH), 5.295-4.693 (1H, dd,-CH-OH), 5.983 (1H, s, -CH-O-C-), 4.597 (1H,d, -CH2-OH). ESI-MS (m/z): 433.1 (M+1).

Δ^{14}-Triamcinolone Hexacetonide impurity (II)

Δ^{14}-Triamcinolone Acetonide (3.3g) was dissolved in 49.5ml methylene dichloride along with 2.6ml Triethyl amine. 3, 3-Dimethyl butanoyl chloride was added to it, allowing exotherm upto reflux. Reaction progress was monitored by TLC. After completion of reaction by TLC, the reaction mass was washed with aqueous Hydrochloric acid solution, followed by aqueous Potassium carbonate solution. Finally, organic layer was subjected for complete solvent distillation and subsequent purification using methanol to get 3.7g Δ^{14}-Triamcinolone Hexacetonide impurity. This is further purified by column chromatography to get 1.5g Δ^{14}-Triamcinolone Hexacetonide impurity pure.IR (KBr): -OH str. (3323cm^{-1}), C=O ketone (1731cm^{-1}), C=C Str. (1660cm^{-1}), C=O ring str. (1617cm^{-1}), C-C ring str. (1370cm^{-1}), C-O str. (1070cm^{-1}). ^{1}HNMR (δ, DMSO): 7.135-7.147 (1H, d, -CH=CH-C=O), 6.369-6.333 (1H, m, -CH=CH-C=O), 6.153-6.139 (1H, d, CH=CH-C=O), 5.007-4.994 (1H, m, -CH –OH [11-OH], 4.963-4.885 (2H, dd, -CH2-O-CO-CH-), 4.600 (1H, s, -CH-OH- (11-OH), 1.432 (3H,s, -CH3 of Acetonide), 1.356 (3H,s, -CH3 of Acetonide), 1.09 [12H, s,-CH2-(CH3) 3- + -CH3 of C18). ESI-MS (m/z): 531 (M+1).

ANALYTICAL

New superior (In-house) HPLC method development

Various parameters of new developed HPLC method are mentioned in Table No.1, 2 and No.3.

SPECTROSCOPY

IR spectroscopy

Details of instrument and ID

Make: Bruker,
Model: Alfa with OPUS-1.2.139.1294 software.
ID: AVIK/QC/I/26

Method of Analysis

Take 200 to 300 mg of previously dried KBr at 105ºC for 2hr into a clean and dry mortar. Triturate to a fine powder with a clean and dry pestle and place in the sample cup. Make the powder surface uniform using sample-pressing bar. Mount this sample cup to the sample cup holder in instrument and Record the background spectrum. Repeat the experiment taking about 2 to 3mg of sample along with the KBr, and record the sample spectrum.

LC-MS/MS Spectroscopy

a. Instrument Name and make Thermo fisher and LCQ fleet
b. Method - Electrospray ionization with (Ion Trap) mass analyzer
c. Software -Xcalibur

Proton NMR Spectroscopy

a. Instrument Model: JNM-ECZ400S/L1
b. Instrument Make: JEOL
c. Software: Delta
d. Proton NMR method of analysis: About 8-10 mg of sample was taken into clean NMR tube and diluted with the deuterated solvent.

RESULTS AND DISCUSSION

The present innovation, started with an unknown factor affecting the purification process during the synthesis of the drug substance, Triamcinolone and its Acetonide derivatives, because of which Impurity B of Triamcinolone Hexacetonide was not getting removed in some instance. The problem solving procedures, then, resulted in the identification of an overlapping unknown impurity and known impurity-B. After identification of the same, on the basis of LC-MS (Figure No.2), Δ^{14}-Impurity has been recognized as an overlapping impurity, with the same RRT (Relative Retention Time), of Impurity-B of Triamcinolone Hexacetonide as per European pharmacopoeia (EP-9.0). Furthermore, Δ^{14} impurity Triamcinolone Hexacetonide impurity has been synthesized and characterized in the laboratory, too.

Though the characterization of steroidal moieties is complex, Δ^{14}-Triamcinolone Hexacetonide impurity has been characterized by the help of spectral studies like IR, Proton NMR, and ESI-MS.

Additionally, most important thing is that New (In-house) superior qualitative HPLC method compare to European Pharmacopoeia (EP-9.0) has been developed successfully to get separation of Δ^{14} Triamcinolone Hexacetonide impurity from impurity-B and API (Triamcinolone Hexacetonide).

Comparison of New developed method with EP-9.0 method is demonstrated in Table No.1, 2 and No.3

Spike study has been performed, to evaluate Δ^{14} impurity of Triamcinolone Hexacetonide, too (Figure No.3).

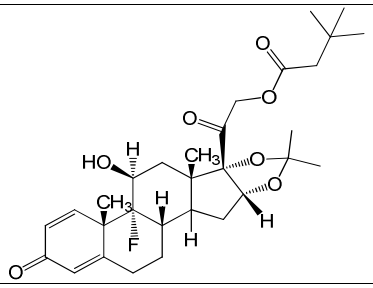
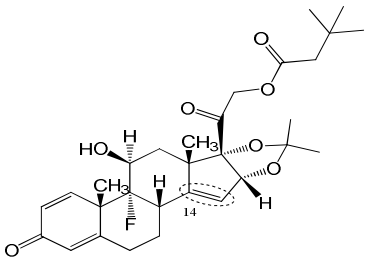
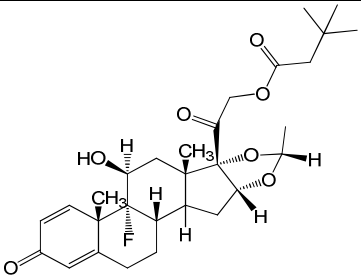
		
Triamcinolone Hexacetonide [TMHA] [I]	Δ^{14}-Triamcinolone Hexacetonide [II]	Triamcinolone Hexacetonide (Impurity-B) (EP-9.0) [III]
9α-fluoro-11β-hydroxy-2', 2'-dimethyl-3, 20-dioxo-(16βH)-[1, 3] dioxolo [4', 5': 16, 17] pregna-1, 4-dien-21-yl-3, 3-dimethyl butanoate	9α-fluoro-11β-hydroxy-2', 2'-dimethyl-3, 20-dioxo-(16βH)-[1, 3] dioxolo [4', 5': 16, 17] pregna-1, 4, 14-trien-21-yl-3, 3-dimethyl butanoate	9α-fluoro-11β-hydroxy-2'-methyl-3, 20-dioxo-(16βH)-[1, 3] dioxolo [4', 5': 16, 17] pregna-1, 4-dien-21-yl-3, 3-dimethyl butanoate

Table No.1: Comparison of Existing Ph. Eur. 9.0 monograph method and In-house superior method

S.No	Key Factor	Existing Ph. Eur. 9.0 monograph method	In-house superior method
1	Specificity	Δ^{14}-TMHA impurity is merging with specified impurity-B; hence resolution obtained was very less between the two impurities.	Δ^{14}-TMHA impurity is well separated from specified impurity-B and resolution obtained was about 2.5 in system suitability solution.
		Triamcinolone Acetonide, TMCA-21-Acetate, Δ^{14}-TMHA, and Impurity-C (unspecified) are the impurities which are not mentioned in EP 9.0 monograph.	Triamcinolone Acetonide, TMCA-21-Acetate, Δ^{14}-TMHA and Impurity-C are possible impurities in route of synthesis and all impurities are well separated from each other.

Table No.2: Comparison of Chromatographic conditions of related substances method

Parameters	Existing Ph. Eur. 9.0 monograph method (Related substances by HPLC)	In-house superior method (Related substances by HPLC)	Change observed
Column	Base deactivated end-capped octadecylsilyl silica gel for chromatography (5 µm)	Thermo Scientific Syncronis C18 (250mm x 4.6mm x 5.0µm)	No change
Mode	Isocratic	Isocratic	No change
Mobile phase	Water: Methanol- (25:75) % v/v	Water: Acetonitrile- (30:70) %v/v	Modified
Column oven temperature	Not mentioned	35˚C	Modified
Sample temperature	Not mentioned	25˚C	Incorporated for betterment of the method
Injection volume	20µl	10µl	Modified
Flow rate	2.0 ml/min	1.0 ml/min	Modified
Wavelength	254 nm	254 nm	No change
Diluent	Methanol and mobile phase	Methanol	No change
Test solution (a)	Concentration about 2.5 mg/ml in methanol	Concentration about 2 mg/ml in methanol	Modified

Parameters	Existing Ph. Eur. 9.0 monograph method (Related substances by HPLC)	In-house superior method (Related substances by HPLC)	Change observed
Reference solution (a)	Dissolve 5mg of Triamcinolone hexacetonide for system suitability CRS (containing impurities B and C) in methanol and dilute to 2.0ml with same solvent.	Triamcinolone Hexacetonide standard, Δ^{14}-TMHA impurity standard, impurity-B standard and Impurity-C standard about 2000µg/ml, 3µg/ml, 3µg/ml and 3µg/ml respectively for system suitability.	Modified to incorporate other possible impurities
Reference solution (b)	Concentration about 2.5µg/ml in mobile phase	Concentration about 2µg/ml in diluent	Modified
System suitability parameters	**Resolution:** Minimum 4.0 between the peaks due to Triamcinolone Hexacetonide and impurity C.	**Resolution:** From the reference solution (a), resolution between peaks due to Δ^{14}-TMHA and Impurity-B is not less than 1.5. **Resolution:** From the reference solution (a) resolution between peaks due to Triamcinolone Hexacetonide and Impurity-C is not less than 3.5. Relative standard deviation of area response of six replicate injections determined from the reference solution (b) for Triamcinolone Hexacetonide is not more than 5.0%	Incorporated system suitability criteria for betterment of the method

Table No.3: Specification limit of existing EP 9.0 monograph method and in-house superior method

Existing Ph. Eur. 9.0 monograph method			In-house superior method		
Name of impurity	**Relative retention time**	**Specification limit (%w/w)**	**Name of impurity**	**Relative retention time**	**Specification limit (%w/w)**
Impurity-B	0.79 min (Epimer-1) 0.81 min (Epimer-2)	Not more than 0.15%	Impurity-B	0.79	Not more than 0.15%
			Possible impurities in route of synthesis not mentioned in EP 9.0 monograph		
			Triamcinolone	0.18	Not more than 0.15%
			Impurity-A (Triamcinolone Acetonide)	0.24	Not more than 0.15%
			TMCA-21-Acetate	0.36	Not more than 0.15%
			Δ^{14}-TMHA	0.84	Not more than 0.15%
			Impurity-C	1.23	Not more than 0.15%
Unspecified impurity	---	Not more than 0.10%	Unspecified impurity	---	Not more than 0.10%
Total impurities	---	Not more than 0.3%	Total impurities	---	Not more than 0.3%

Note: We have already accomplished analytical method validation of related substance by HPLC using above mention in-house chromatographic condition, parameters and impurities.

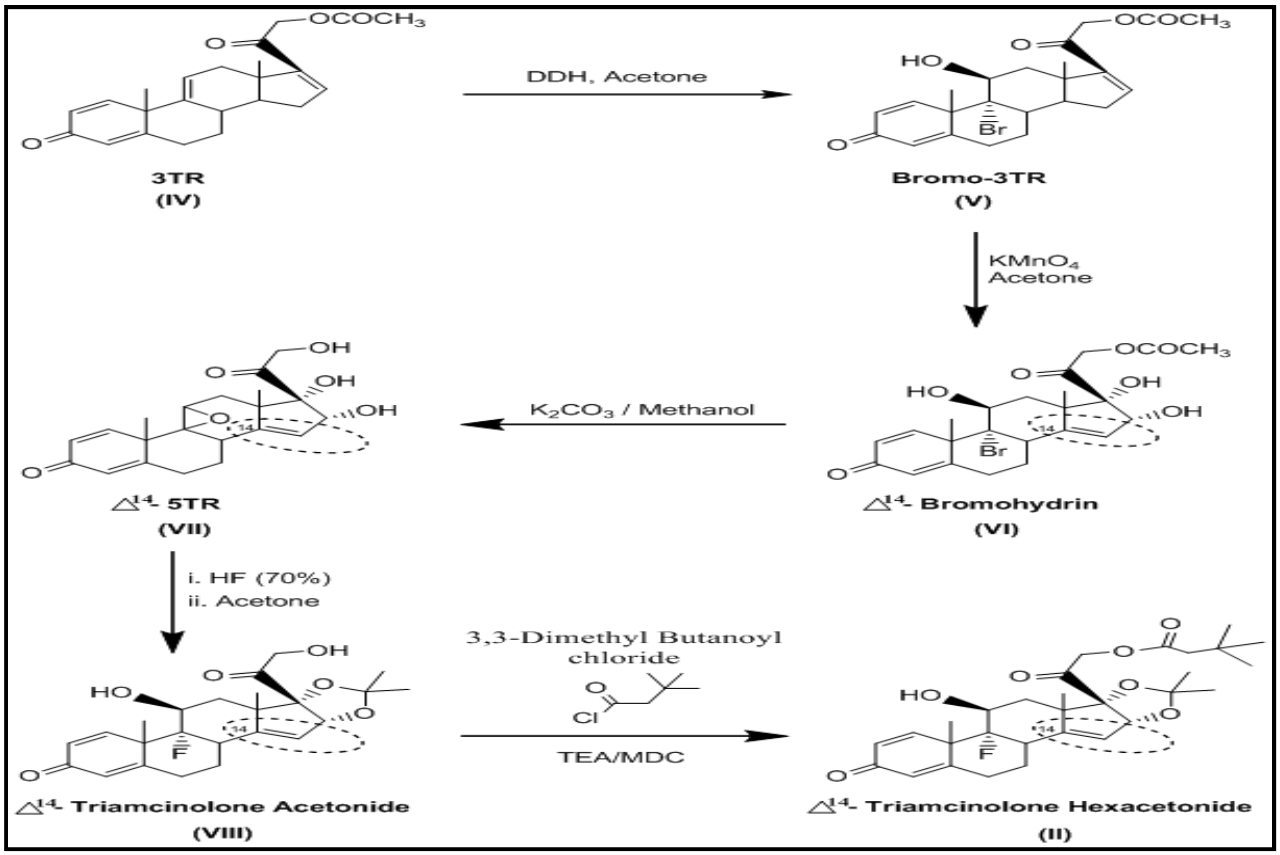


Figure No.1: Reaction Scheme: Route of synthesis of Δ14-Triamcinolone Hexacetonide

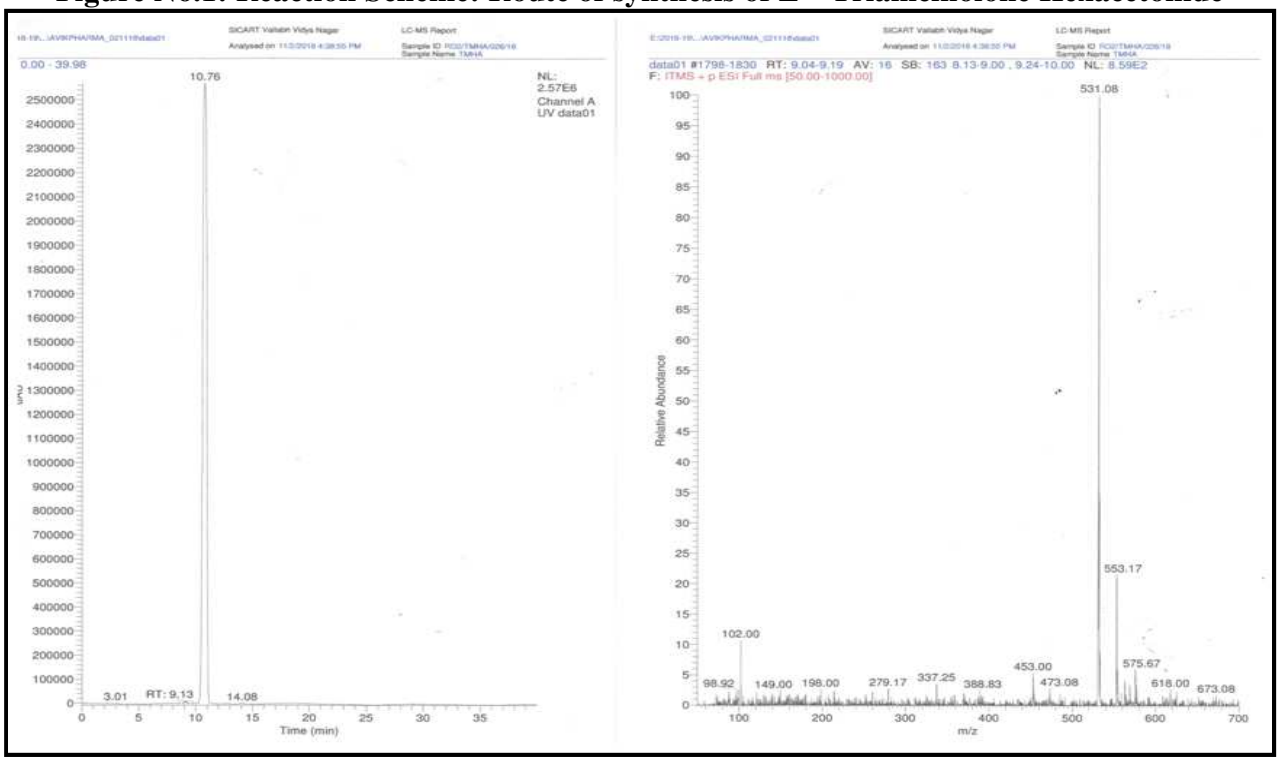
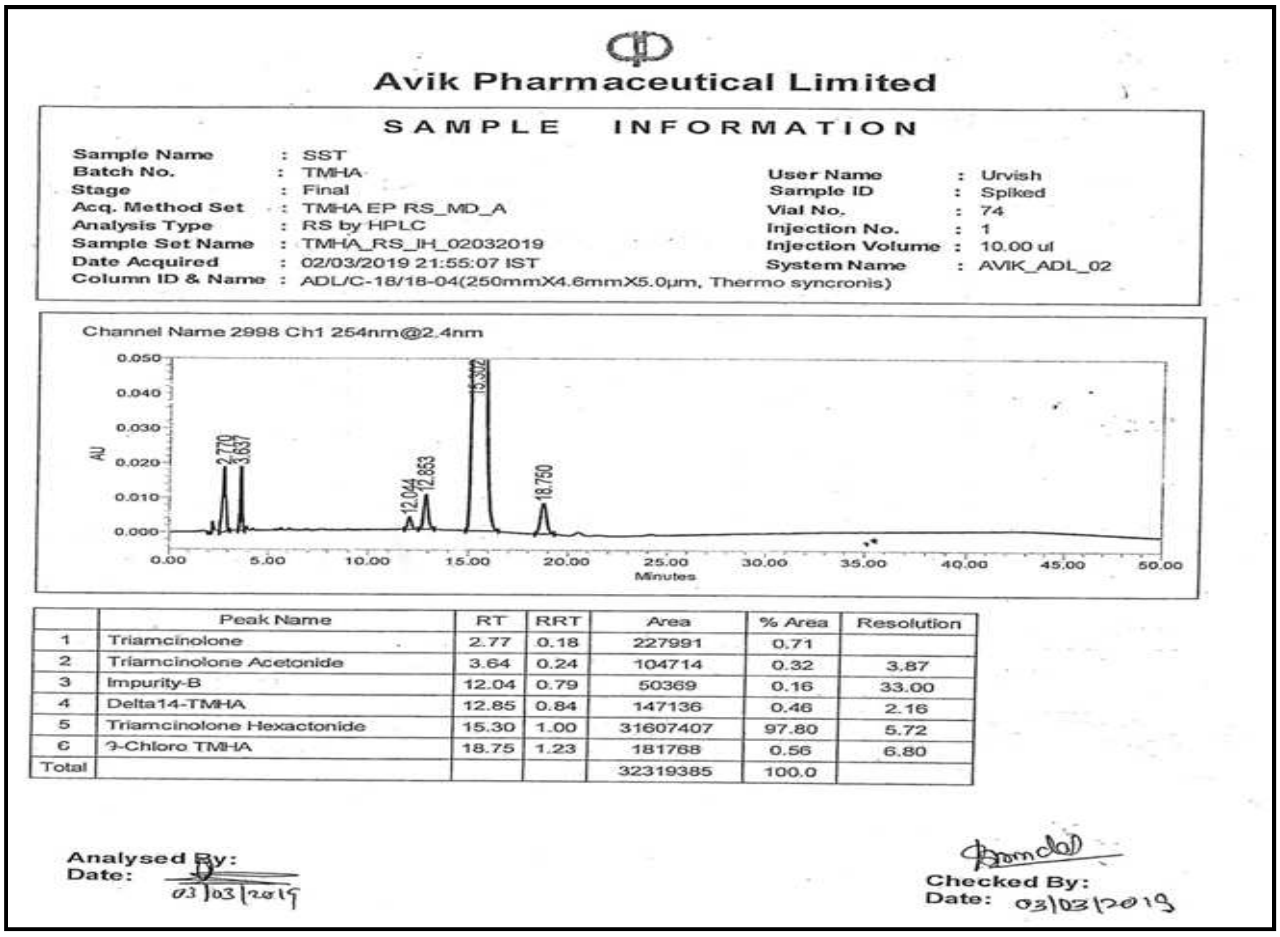


Figure No.2: LC-MS of 0.79 RRT impurity in Triamcinolone Hexacetonide (TMHA)



	Peak Name	RT	RRT	Area	% Area	Resolution
1	Triamcinolone	2.77	0.18	227991	0.71	
2	Triamcinolone Acetonide	3.64	0.24	104714	0.32	3.87
3	Impurity-B	12.04	0.79	50369	0.16	33.00
4	Delta14-TMHA	12.85	0.84	147136	0.46	2.16
5	Triamcinolone Hexactonide	15.30	1.00	31607407	97.80	5.72
6	9-Chloro TMHA	18.75	1.23	181768	0.56	6.80
Total				32319385	100.0	

Figure No.3: Spike study of all impurity along with Δ^{14}-Impurity in Triamcinolone Hexacetonide

CONCLUSION

All the specified impurities including possible impurities from route of synthesis (Triamcinolone, Impurity-A, TMCA-21-Acetate, Impurity-B, Δ^{14}-TMHA and Impurity-C) are well separated from each other; hence the in-house developed method is superior to the existing Ph. Eur. 9.0 monograph method. Secondly, as per ICH guidelines[6], the present process also sanctions limit of Δ^{14}-Triamcinolone Hexacetonide to be increased from 0.10% (Unspecified) to 0.15% (Specified) as it is now known. The increased limit of Δ^{14}-Triamcinolone Hexacetonide helps to qualify the material as per stringent specification of EP-9.0 which is very difficult to reduce in purifications. Therefore control of Δ^{14}-Triamcinolone Hexacetonide at synthesis stage is very important for quality perspective of final Active Pharmaceutical Ingredient (API). Thirdly, we have identified that the root cause of generation of Δ^{14} impurity was from its Key Starting Material (5TR) which was prepared the same Δ^{14}-Impurity of 9,11β-Epoxide Triamcinolone (5TR), Triamcinolone Acetonide (TMCA) and Triamcinolone Hexacetonide (TMHA), characterized and confirmed.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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