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DEVELOPMENT AND VALIDATION OF REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) METHOD FOR ESTIMATION OF TRIAMCINOLONE ACETONIDE (KENACORT A-40) IN BULK AND ITS PHARMACEUTICAL FORMULATION

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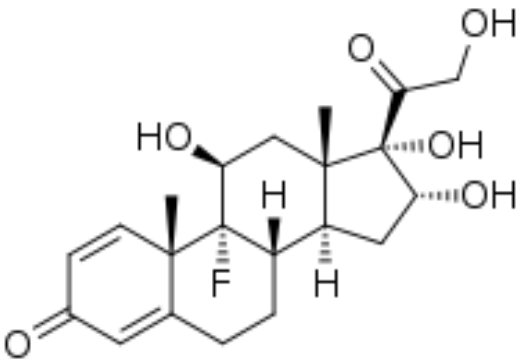
Abstract

A straightforward and precise Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) for the estimation of triamcinolone Acetonide was developed. Methanol: water mixture was used as the mobile phase. The validation parameters like linearity, accuracy, precision, robustness, the Limit of detection, and the Limit of quantitation were performed for Triamcinolone Acetonide. Concentration was found to be around 25mg/mL. Recovery and assay studies of Ranitidine HCl were 99%, indicating that the proposed method can be adopted for quality control analysis of Ranitidine HCl. Validation of the Method for the drug and its formulation was found to be Precise, Accurate, Selective, and Sensitive. RSD was recorded up to the maximum Limit of 1.302% within the allowed Limit of 2%. Accuracy/Recovery was seen as 100%, 120%. Intermediate Precision was observed as 100%, while reproducibility was found as 100%. Co-relation Co-efficient & Y-intercept are also within the Limit of 0.9988 & 0.2512 with linear graph.

Keywords:
Method Development, Validation, R.P.- HPLC, Triamcinolone Acetonide.

Introduction

Triamcinolone Acetonide Injection is sold under the brand name Kenacort-A 40 and is used to treat various allergies, inflammation, arthritis, and various skin diseases (Elks, 2014). It is injected deep into muscles to treat these symptoms. The molecular formula of Triamcinolone Acetonide is $C_{24}H_{31}O_6$, and its molecular mass is 434.504 g/mol. Its chemical structure is given in Figure 1.



9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione

Figure1. Chemical Structure of Triamcinolone Acetonide

Analytical method development and validation are essential components in drug development for identification, quantitation, and determination of the purity of the drug compound. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies, which mainly concern the in-process controls during the manufacturing of the drugs; it ensures that the drug must be safe, effective, and acceptable to the patients use (Supriya et al., 2018, Aluka et al., 2015, Eduardo et al., 2018, Bhagya Laxmi et al., 2018).Spectroscopy and chromatography are two important tools in the process of method development and validation. For drug analysis, pharmaceutical industries mostly use both techniques because these are highly sensitive and selective to monitor even a minimum quantity of the material and easily detect known and unknown impurities (Nidhi et al., 2018; Dewani et al., 2015; Gaudla et al., 2016).

These methods are not only used in pharmaceutical industries but also used in food and other industries. Thus, HPLC and spectroscopy-based methods have vital scope and use in almost all kind of chemical industries and are increasing day by day. In pharmaceutical industries mostly, RP-HPLC is used with suitable column compositions like C8, C.N., and C18 with organic solvents like methanol, acetonitrile, and buffers (Farah et al., 2016; Nareddy et al., 2015; Madhusudhan et al., 2015, Lakshmi et al., 2015).
After drug method development, it is necessary to validate all the parameters that were set in the method development, for example, robustness and reproducibility. Method validation focuses on process criticality and regulatory requirements, and its protocols are monitored by many authorities like the Food & Drug Administration (FDA) and the International Conference on Harmonization (ICH) (Martindale 2007, U.S Pharmacopaea 2007, Eur. Pharmacopaea 2008, British Pharmacopaea 2005).

1. Materials and Methods

1.1.Chemicals and reagents

The chemicals used were methanol, deionized water, Buffer (Ammonium acetate), Triamcinolone Acetonide (as standard), and Kenacort injection (as sample). The chemicals used were of HPLC grade and were used without further purification. Kenacort Injection was purchased from the local market, whereas Glaxo SmithKline laboratories in Karachi gifted pure Triamcinolone Acetonide.

1.2.Instrumentation

The spectroscopic analysis was performed on a double-beam spectrophotometer (SL218, Elice). The High-Performance Liquid Chromatography (HPLC)experiments were carried out on an isocratic reversed-phase HPLC, Agilent 1200, with a UV detector and 200mm x 4.6mm id. Stainless steel column packed with 10µ Spheris orb ODS 1.

2.3.1 Standard Preparation

An amount of 22.4mg Triamcinolone Acetonide in pure form was taken into a 200 volumetric flask, then dissolved and diluted up to volume with mobile phase.

2.3.2 Sample Preparation

Triamcinolone Acetonide(5ml) was placed in a 250ml volumetric flask and diluted to volume with the mobile phase. Further transfer 5ml of diluted solution to 25ml volumetric flask and diluted to volume with mobile phase.

2.3.3 Mobile Phase composition

To a mixture of methanol: water (98:2) was added 50ml of 2-Propanol, 300ml of 0.05M of ammonium acetate, and 650ml of deionized water. The phrase adjusted to 4.5 $\pm$ 0.05 with dilute acetic acid.

2.3.4 Stationary phase composition

The stationary phase consists of a 200mm x 4.6mm id. Stainless steel column packed with 10µ Spheris orb ODS.

2.4.Chromatographic conditions

Kenacort Injection is soluble in methanol and water, so was made the mobile phase of 98% methanol and 2% DI water and 1g of ammonium acetate was added as buffer. For UV/Vis detection, the wavelength range was set at 700 to 200 nm, and the chromatograms were recorded at different wavelengths of maximum absorption, i.e., 325, 279, and 224nm, showing better results at 325nm.

In the RP-HPLC method, the retention time for Triamcinolone Acetonide was set to 2-4min, and the flow rate was 2ml/min. The temperature of the column was maintained at 30^0C, and the wavelength of the UV detector was set at 325nm. A volume of 50 µl was injected into the system for analysis.

3. RESULTS AND DISCUSSION

The optimized chromatographic conditions were applied throughout the method validation process, which provided fine resolution of the triamcinolone acetone in pure and dosage forms. The retention time recorded for the sample and standard at 325nm is given in Figure 2.

Fig.2 (a)Standard Chromatogram (b) Sample Chromatogram

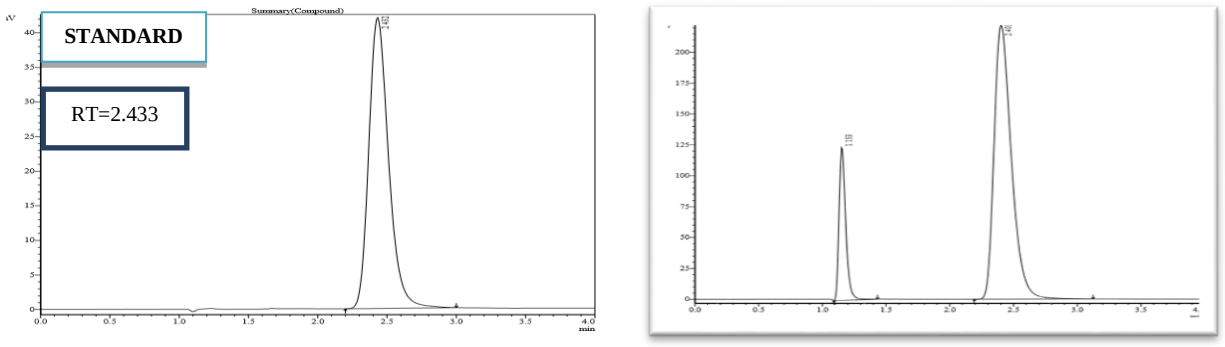


Figure No.2(b)In the sample solution, there are two peaks at RT 1.14 and 2.44 nm. The second peak is comparable with the standard peak, confirming the active ingredient, whereas the first peak is any unknown impurity or excipient.

3.1. VALIDATION OF HPLC METHOD

The RP-HPLC method was then validated as per standard protocols, and the results are reported below.

3.1.1. System Suitability

System Suitability is performed to confirm that the system and reference material are suitable for further analysis (Arun et al., 2012). Two standard solutions, A and B, were prepared, and five runs from A and three runs from B were performed according to the method. **%**RSD calculated by peak area complies with the acceptance criteria indicating the suitability of the system: Ref. Std. 1-3 RSD≤ 0.85 and Ref. Std. 1-5 RSD≤ 1.5% (Table No.1)

Table No.1

Injection #	Peak Area	Statistical Analysis
Ref: Std: A#1	998732	**System Precision:** Ref: Std. 1-3 RSD = 0.192% Ref: Std. 1-5 RSD=1.29%
Ref: Std: A#2	1013809	
Ref: Std: A#3	1024484	
Ref: Std: A#4	1027396	
Ref: Std: A#5	1032200	
Ref: Std: B#1	1039713	
Ref: Std: B#2	1041613	
Ref: Std: B#3	1043580	

3.1.2. Precision (Repeatability)

The authenticity of the method is measured in terms of precision, which is the ability of the method and system to reproduce the same results (Venkatesan et al., 2014).

For this purpose, six replicates were prepared as per the method and injected in duplicate runs of each sample. The results obtained are tabulated in Table no.2, which shows that the RS calculated is within the acceptable Limit, that is ≤ 2.0 %.

Table No.2

Preparation	Injection	Result (mg)	Statistical Analysis %age	
1	1	25.038	100.152	**Mean:100.10% SD:0.093 RSD % :0.095**
	2	25.035	100.14	
2	3	25.030	100.12	
	4	25.049	100.196	
3	5	25.077	100.308	
	6	25.086	100.344	
4	7	25.033	100.132	
	8	25.034	100.136	
5	9	25.011	100.044	
	10	25.015	100.06	
6	11	25.054	100.216	
	12	**25.066**	**100.264**	

3.1.3. Intermediate Precision

Intermediate precision was calculated following the above procedure, and the values obtained are given in Table No. 3. The Results obtained for intermediate precision meet the acceptance criteria of RSD≤ 2.0 %.

Table No,3

Preparation	Injection	Results (mg)	Average Result (mg)	Statistical Analysis %age	
1	1	25.009	25.0145	100.058	**Mean:100.05%** **SD:0.111** **RSD % :0.121**
	2	25.020			
2	3	25.021	25.0255	100.102	
	4	25.030			
3	5	25.069	25.078	100.312	
	6	25.087			
4	7	25.015	25.0075	100.03	
	8	25.000			
5	9	25.006	25.006	100.024	
	10	25.006			
6	11	25.013	25.0085	100.034	
	12	**25.004**			

3.1.4. Accuracy/Recovery

Accuracy/Recovery is calculated by taking a placebo first and adding a reference standard to it (Nakashina 2005). For this purpose, three samples of the active ingredient into placebo at 80%, 100%, and 120% were prepared and injected in triplicate, resulting in 09 injections of each of the concentrations, and a total of 27 injections were performed. Table 4 shows that the results of recovery are within the acceptance criteria: 98-102%.

Table No.4

Input Level%	Ranitidine HCl added (mg)		Ranitidine HCl recovered (mg)		% Recovery		Statistical Analysis
80	1	17.92	1	20.060	1	80.24	
				20.062		80.248	
				20.091		80.364	
	2	17.92	2	20.140	2	80.056	**X:80.33%** **SD:0.207** **% RSD:0.251**
				20.140		80.56	
				20.138		80.552	
	3	17.92	3	20.144	3	80.576	
				20.144		80.576	
				20.171		80.684	
100	1	22.4	1	25.029	1	100.116	
				25.095		100.38	
				25.091		100.364	**X:100.322** **SD:0.148**
	2		2	25.007	2	100.028	

		22.4		25.008		100.032	**% RSD:0.144**
				25.031		100.124	
	3	22.4	3	25.084	3	100.336	
				25.070		100.28	
				25.090		100.36	
120	1	26.88	1	30.073	1	120.292	**X:120.45% SD:0.945% %RSD:0.776**
				29.853		120.019	
				30.102		120.007	
	2	26.88	2	29.973	2	119.892	
				29.993		119.972	
				30.003		120.012	
	3	26.88	3	30.026	3	120.104	
				30.082		120.328	
				30.079		**120.316**	

3.1.5. Linearity

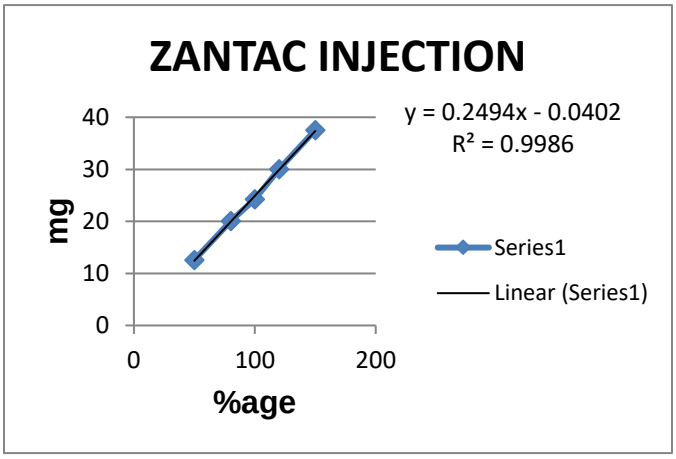
Linearity is the ability of the procedure to obtain test results within its given range. Those results are directly proportional to its concentration, which is checked by the linearity graph, its Correlation Coefficient R^2, and Y-intercept with slope. For the calculation of linearity, three replicates of each analyte concentration 50%, 80%,100%, 120%, and 150% were prepared according to the conditions of the analytical method, which produced the following results in table 5.

Table No.5

Concentration (%)	**Result (mg)**	**%age**	**Statistical Analysis**
50	12.571	50.284	Correlation Coefficient $r^2 = 0.998$ Y-intercept == 0.2494x - 0.0412
	12.597	50.388	
	12.564	50.256	
80	20.045	80.18	
	20.085	80.34	
	20.097	80.388	
100	24.335	97.34	
	24.228	96.912	
	24.254	97.016	
120	30.003	120.012	
	30.000	120.00	
	30.061	120.244	
150	37.558	150.232	
	37.518	150.072	
	37.506	150.024	

Linearity complies with the acceptance criteria, i.e., Correlation Co-efficient: $r^2 \geq 0.9997$ ($r^2 \geq 0.999$), Y Intercept: $\leq 2.0\%$. The results are represented in graphical form (Figure 3), showing the linearity of the method.

Figure No.03



1.1.6. Robustness

Robustness is the capacity of the procedure to remain unaffected by small changes like changes in temperature, wavelength flow rate, etc. The sample was prepared according to a described method and was injected by changing the following parameters.

- Change in λ wavelength ± 2nm.
- Change in column temperature ± 2˚C
- Change in flow rate ± 0.2ml/min

The results obtained (Table 6) demonstrate that the method is robust and complies with the acceptance criteria, with a difference of ≤ 2.0 %.

Table No. 6

S.NO.	Parameters	Result (mg)	% Difference
1	STD λ wavelength 325 nm	25.002	0.008%
2	λ wavelength 327 nm	25.002	0.008%
3	λ wavelength 323 nm	25.016	0.064%
1	STD Column temperature 30°C	25.003	0.012%
2	Column Temperature 32 °C	25.001	0.004%
3	Column Temperature 28 °C	25.016	0.064%
1	STD Flow rate 2 ml/min.	25.035	0.14%
2	Flow rate 2.2 ml/min.	25.056	0.0224%
3	Flow rate 1.8 ml/min.	25.018	0.072%

4. Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The Limit of quantitation (LOQ) and Limit of detection (LOD) were obtained from signal to noise ratio, which was based on a standard deviation of the slope of the calibration curve and response (Katarzyna et al., 2010; Beata et al., 2009). The calculation of LOQ and LOD is given below.

4.1. Limit of Quantitation (LOQ)

The following formula determines the Limit of quantitation

LOQ = 10[A]/(S/N)

- Where: [A] = Concentration of analyte in the sample
- S/N = 2H/h

- 10 is a factor of signal-to-noise
- Where: H = Height of analyte peak
- h = Height of the noise (range between minimum and maximum noise value).

Placebo solution Reading

Solutions	Upper (Height)	Lower (Height)	Height
Placebo - 01	75.46	135.83	211.29

- Placebo maximum height 211.29
- H= height of analyte peak in reference solution in any one injection (= H) =905593
- h= maximum noise (= h) = 211.29

Formula to Calculate Signal to Noise ratio S/N = 2H/h

- H= h x S/N = (211.29*13)/2 = 1373.385
- S/N = 2*1373.385/ 211.29 = 13
- A= (224*1373.385)/905593 = 0.3397

Theoretical calculation of Active in reference standard A= (22.4/100*1000*3/2000) =0.336ppm

LOQ SOLUTION

H	**h**	**A**	**S/N = 2H/h**	**LOQ = 10[A]/(S/N)**
1373.38	211.29	0.33397	13	ppm

The calculated value for LOQ is 0.261ppm, which is the lowest amount to be quantified.

4.2. Limit of Detection (LOD)

LOD can be calculated by following the formula

LOD = 3[A]/(S/N)

- Where: [A] = Concentration of analyte in the sample
- S/N = 2H/h
- 3 is a factor of signal-to-noise
- Where: H = Height of analyte peak
- h = Height of the noise (range between minimum and maximum noise value).

Placebo solution Reading

Solutions	**Upper (Height)**	**Lower (Height)**	**Height**
Placebo - 01	75.46	135.83	211.29

- Placebo maximum height 211.29
- H= height of analyte peak in reference solution in any one injection (= H) =905593

- h= maximum noise (= h) = 211.29

Formula to Calculate Signal to Noise ratio S/N = 2H/h

- H= h x S/N = (211.29*3.5)/2 = 369.75
- S/N = 2*369.7575/ 211.29 = 3.5
- A= (224*369.75)/905593 = 0.0915

Theoretical calculation of Active in reference standard A= (22.4/100*1000*3/2000*27/100) =0.0907ppm

LOD SOLUTION

H	**h**	**A**	**S/N = 2H/h**	**LOD = 3[A]/(S/N)**	
369.75	211.29	0.0915	3.5	0.078	ppm

LOD calculated is 0.078ppm, which is said to be the lowest amount to be detected under optimized conditions.

5. Conclusion

A Simple, selective, precise, and accurate reverse-phase HPLC method for the estimation of triamcinolone acetone (Kenacort Injection) in pure and dosage form was developed according to ICH guidelines. The method was validated for linearity, intermediate precision, repeatability, recovery, robustness, and system suitability. %RSD for precision and accuracy of the method was found to be less than 2%, which shows that the method has a high degree of precision.

The correlation coefficient was calculated to be 0.998, which demonstrates the superb linearity of the method. The LOD and LOQ for Zantac Injection were 0.0392ppm and 0.1307ppm, respectively. The method exhibited good resolution of peaks with rational retention time. Hence, the method can be used for routine analysis of pharmaceutical agents with reliability and accuracy.

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