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DEVELOPMENT AND VALIDATION OF REVERSE PHASE LIQUID CHROMATOGRAPHY METHOD FOR ESTIMATION OF ISOTRETINOIN (13-CIS RETINOIC ACID) IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

In the present study, a reverse phase high performance liquid chromatographic method was developed and validated for the determination of Isotretinoin in pharmaceutical dosage form. Chromatographic separation was carried out on a C-8 column using a mobile phase consisting of acetonitrile:isopropyl alcohol (50:50, v/v) adjusted at pH 5.0 using 1% ortho phosphoric acid. Flow rate was 1ml min−1 and UV detection was carried at 280 nm. Caffeine was used as an internal standard. The calibration curve was linear over the range 5–600µgml−1. R.S.D. for precision study was found to be <1%. The result of accuracy study was ranged between 98.61% and 101.51% with a R.S.D. lower than 2%. LOD and LOQ were found to be 0.0428µgml−1 and 0.1298µgml−1, respectively. The method was simple, rapid, easy to apply and very suitable for routine analysis of Isotretinoin in pharmaceutical dosage form.

Keywords: Isotretinoin, Caffeine, RP-HPLC, Internal Standard,13-Cis Retinoic Acid.

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INTRODUCTION

Isotretinion (IST), 13-cis isomer of retinoic acid, is a retinoid classified as vitamin A. Isotretinoin is a topical keratolytic agent which is used in the treatment of skin diseases including acne vulgaris. The mechanism of action is believed to inhibit the secretion of sebum and alter the lipid composition of the skin surface[1]. Pure crystalline isotretinoin is a yellow-orange powder whose faint odour resembles that of vitamin A. It is soluble in chloroform and methylene chloride, sparingly soluble in ethanol, 2-propanol, polyethylene glycol and very sparingly soluble in water[2].

Assay of Isotretinoin is performed by titrating with 0.1N sodium methoxide in USP[3] whereas potentiometric titration with tetrabutyl ammonium hydroxide is reported in BP[4]. In previous studies, Isotretinoin was determined by gas chromatography in soft and hard gelatine capsules[5]. HPLC method for the simultaneous determination of tretinoin and isotretinoin is also reported[6]. Tretinoin (13-trans retinoic acid) and retinoid were determined in different formulations by reversed-phase HPLC[7,8,9]. It was revealed that none of RP-HPLC method reported for the determination of IST using an internal standard (IS). The uncertainties introduced by sample injection can be avoided by use of IS in quantitative chromatography. Hence an aim of this work was to develop a rapid, precise, accurate and comparatively economical RP-HPLC method with UV detection for quantitative estimation of IST in soft gelatine capsule dosage form. The results obtained have been statistically validated in accordance with the ICH guidelines[10].

EXPERIMENTAL

Reagents and Chemicals

All the reagents like ortho phosphoric acid, acetonitrile (Qualigens fine chemicals, Mumbai) and water used were of HPLC grade. IST and Caffeine (CAF) standards were obtained from Strides Arco

Laboratories (Bangalore, India) and Juggat Pharma (Bangalore, India) respectively. The marketed formulation (Tufacne-20) was purchased from the local pharmacy.

Instrumentation

The HPLC system used was Shimadzu LC-20AT pump, Rheodyne injector (20µl), SPD-20A UV detector and the system was controlled through Spinchrome software. Analytical column used for this method was PHENOMENEX Luna C8 (250X4.6mm, 5µ). Sartorius digital Balance was used for weighing and Digital pH meter 7007 for the pH measurement.

Chromatographic conditions

The composition of the mobile phase was acetonitrile:isopropyl alcohol (50:50, v/v) (adjusted to pH 5.0 with 1% orthophosphoric acid). The mobile phase was vacuum-filtered through 0.2 µm Supor200 membrane and degassed by ultrasonication for 10min before use. The mobile phase flow rate was set at 1 ml min−1. All standard and assay samples were filtered through cellulose acetate (0.45µm) filter before injection. After equilibration of column with the mobile phase indicated by a stable baseline, aliquots of sample (20 µl) were injected and the total run time was kept 20 min. The absorbance of the eluent was monitored at 280nm with a detection sensitivity of 0.1000 aufs. CAF (5µgml−1) was used as an IS.

Standards and sample solutions preparation

Standard stock solutions of IST (1000µgml−1) and CAF (1000µgml−1) were prepared in HPLC grade methanol. Working standard solutions were freshly prepared daily by appropriate dilution of the stock solutions with mobile phase.

Ten soft gelatine capsules were cut with the sharp blade and added in about 50ml of methanol. This was sonicated for 10 to 15 minutes and then filtered by using whatman filter paper no. 41. Extracted solution of soft gelatine capsule was diluted with methanol in 100ml volumetric flask. From the above solution 1 ml was transferred in to 10ml volumetric flask along with 1ml of CAF solution (50 µgml−1) made up to volume with mobile phase (100µgml−1 IST and 5µgml−1 CAF) and results are as given in Table 1.

Method validation

Analytical method validation was carried out under the guidelines of International Conference on Harmonization (ICH). The assay was validated with respect to linearity, precision, accuracy, sensitivity and robustness.

Linearity

Calibration curves were obtained from injecting the six sets of eleven serial different drug concentrations (5, 10, 20, 30, 40, 50, 100, 200, 300, 500, 600µgml−1 of IST). The curves were generated by plotting the peak area ratios between IST and CAF against IST concentration. Linearity was evaluated by linear regression equation.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as relative standard deviation (R.S.D.). Repeatability was determined by performing nine determinations from triplicate injections of three different concentrations of IST (10, 50 and 100µgml−1) on the same day at different time intervals and on three different days for inter-day precision.

Accuracy/recovery

In this study, accuracy was determined based on the recovery (percentage) of known amounts of standard IST added in the assay samples. This was performed by analyzing IST at three concentration levels (50, 100 and 150µgml−1), with a constant concentration of 5µgml−1 of internal standard. Samples were prepared in triplicate. The accuracy of the assay was determined by comparing the found concentration with the added concentration.

Sensitivity

Sensitivity of the method was determined by means of the detection limit (LOD) and quantification limit (LOQ). The LOD and LOQ were measured based on the method described by the International Conference on Harmonization. Calculations for LOD and LOQ were based on the standard deviation of

the calibration curve (σ) and the slope of curve (*S*), using the equation LOD= 3.3×σ/*S* and the equation LOQ= 10×σ/*S*.

Robustness

Robustness of the method was evaluated by the analysis of IST solution under different experimental conditions such as pH of the mobile phase and flow rate. The flow rate was varied 1±0.1 ml min−1 and pH of the mobile phase was changed 5.0 ±0.2 units. Their effects on the retention time (*t*R), tailing factor (*T*) and resolution of the peaks (*R*) were studied.

RESULTS AND DISCUSSION

Optimization of the chromatographic method

The chromatographic conditions were adjusted to provide the best performance of the assay. For system optimization the important parameters such as type and concentration of organic solvents, pH and mobile phase flow rate were investigated.

Effect of mobile phase composition

Different mobile phase composition were tried to achieve better separation and resolution(R) between IST and CAF. It was observed that the acetonitrile:isopropyl alcohol system gave a better resolution and peak symmetry than the methanol:acetonitrile system. Different proportions of acetonitrile:isopropyl alcohol (50:50, 40:60, 30:70, 20:80v/v) were tested and evaluated before the final chromatographic conditions were selected. Finally, acetonitrile:isopropyl alcohol (50:50 v/v) (adjusted to pH 5.0 with1% orthophosphoric acid) was chosen as mobile phase. As a result, the standard solutions of IST and CAF showed symmetric and well-defined peaks, with an average retention time for IST of 3.4 min and 2.9 min for the CAF.

Effect of flow rate

Different mobile phase flow rates (0.9, 1.0 and 1.1mlmin−1) were investigated. The optimum flow rate for which the column plate number (N) was maximum, with the best resolution between all components and with a short run time (<10min) was found to be 1 mlmin−1.

Internal standard

Different compounds were tested as IS for the chromatographic procedure. Among them, CAF eluted before 10 min of the analysis and has a better symmetry and resolution with respect to IST. Therefore, CAF has been chosen as an IS.

Method validation

System suitability

System suitability was performed to confirm that the equipment was adequate for the analysis to be performed. The test was carried out by making six replicate injections of a standard solution containing 10.0μgml−1 and 5.0μgml−1 of IST and CAF (IS), respectively, and analyzing each solute for their peak area, theoretical plates (*N*), resolution(R) and, tailing factor (T). The results of system suitability in comparison with the required limits are shown in table 2. The proposed method fulfils these requirements within the accepted limits.

Linearity

The standard calibration curve was linear over the concentration range 5–600μgml−1. The correlation coefficient obtained after linear regression analysis was 0.9995. The equation of the calibration curve based on the peak ratio of IST/IS with respect to IST concentration was found to be $y = 0.05830x + 0.58306$.

Precision

The R.S.D. of repeatability (intra-day) and intermediate precision (inter-day) ranged between 0.042% and 0.361%. These values show a low variability between the values obtained for each concentration. These values are shown in Table 3.

Accuracy

The results of the accuracy studies are shown in Table 4. Recovery ranged between 98.67% and 101.51% with R.S.D. less than 2%. The values obtained show a suitable accuracy for the analytical method

Sensitivity

LOD and LOQ were 0.042µgml−1 and 0.129µgml−1, respectively. These values are adequate for the detection and quantification of IST.

Robustness

During the robustness study, peak symmetry (T) was maintained and the retention times were not significantly changed as shown in Table 5. These facts suggest that the method did not change with time and experimental conditions. However, it could be noted that organic composition of the mobile phase can influence the method performance.

CONCLUSIONS

In the present research work to achieve highest precision in quantitative chromatography of IST in pharmaceutical dosage form, a RP-HPLC method for IST using IS was developed and validated. The method was validated in terms of linearity, precision, accuracy, detection limit, quantification limit and robustness. It involves a simple procedure for the preparation of the samples and shorter run times for analytical procedure (less than 10 min). Hence the present HPLC method can be considered simple, rapid, suitable and easy to apply for routine analysis of Isotretinoin in pharmaceutical dosage form.

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Table 1: Assay of Isotretinoin Capsule (IST)

Component	Label claim (mg)	Amount found (mg)	Amount found(%)
IST	20	20.58	102.93
	20	20.96	104.81
	20	19.93	99.66
	20	20.96	104.81
	20	19.93	99.66
	20	20.58	102.93
	Mean	20.49	102.45
	%RSD	2.2	2.2

Table 2: System Suitability Results of the Proposed Method.

Analyte	R	N	T	RSD (n=3) tR	RSD (n=3) peak area
CAF		4086	1.652	0.03	0.20
IST	2.917	3796	1.400	0.01	0.24
Required limits	$R > 2$	$N > 2000$	$T < 1.5$	R.S.D. < 5%	

Table 3: Summary of Precision Determined During Method Validation

Concentration (μgml−1)	R.S.D. (%), intra-day	R.S.D. (%),inter-day
10	0.318	0.361
50	0.113	0.008
100	0.042	0.032

Table 4: Accuracy Of The Method Determined According To ICH Q2 Guidelines.

Concentration (μgml−1) Added	Concentration (μgml−1) Recovered	Recovery (%)[a]	R.S.D. (%) (*n*=3)
50	49.35	98.70	
50	49.33	98.67	1.60
50	50.72	101.45	
100	99.88	99.88	
100	99.48	99.48	0.29
100	100.05	100.05	
150	152.27	101.51	
150	150.19	101.12	0.22
150	152.27	101.51	

[a] (Found concentration/added concentration) ×100.

Table 5: Results of Robustness Study.

Parameter	Value	R	T	*t*R (min)	Recovery (%)	R.S.D. (%)
pH	4.8	4.659	1.22	3.593	99.73	0.02
	5.0	3.208	1.52	3.483	100.00	0.11
	5.2	2.494	1.48	3.610	100.44	0.03
Flow rate	0.9	4.659	1.22	4.273	103.1	0.02
(mlmin−1)	1.0	3.208	1.52	3.483	100.0	0.11
	1.1	4.347	1.31	3.900	97.74	0.01

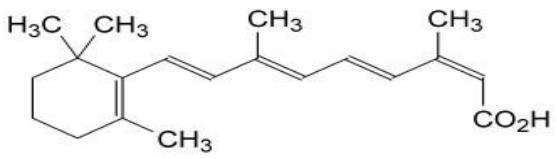

Fig.-1: Chemical structure of isotretinoin (IST)

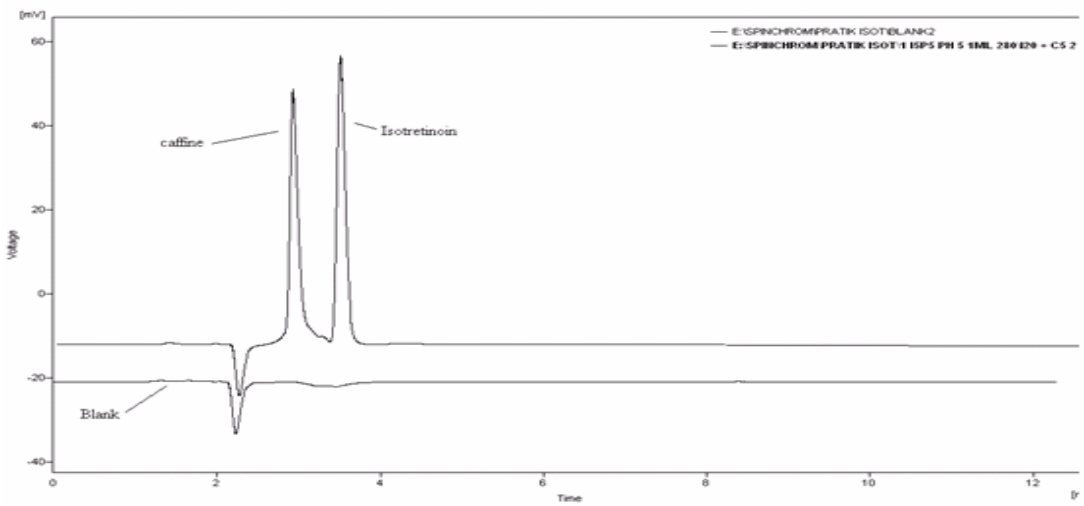

Fig.-2: Overlay Chromatogram of Blank and IST

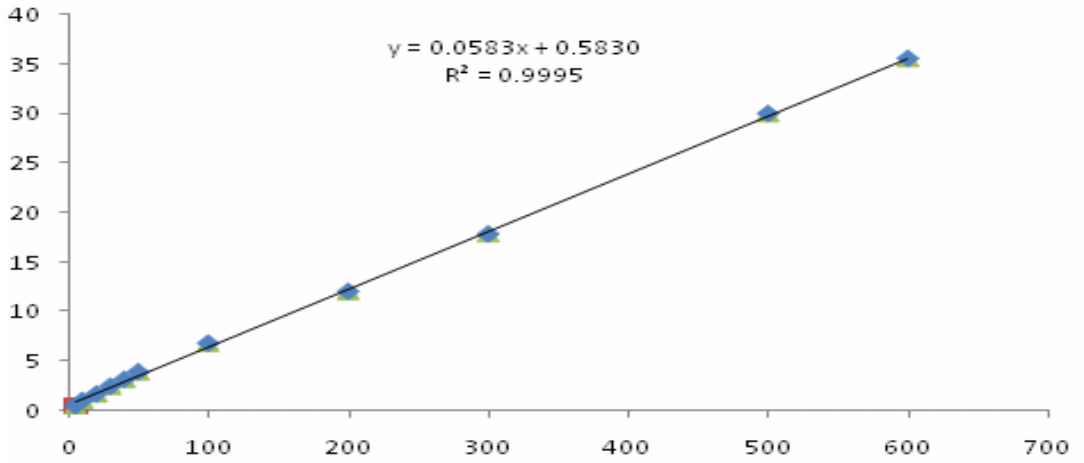

Fig.- 3: Calibration Curve Of Isotretinoin

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